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THE EFFECT OF SALINITY ON THE AMINO ACID CONCENTRATION IN RANGIA CUNEATA (PELECYPODA)

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Simpson *et al.* (1959) reported that the amino acid taurine was in high concentration in marine molluscs. Taurine could not be detected by means of paper chromatography in extracts of fresh-water and terrestrial molluscs. Allen and Awapara (1960) studied the metabolism of S-35 methionine in the mussel, *Mytilus edulis*, and the brackish-water clam, *Rangia cuneata*. Taurine was not detectable in the tissues of the latter. Following injections of radioactive methionine, both species were found capable of degrading methionine to taurine and sulfate. In *R. cuneata* taurine was formed but was rapidly metabolized and excreted as an unknown compound. Taurine was retained in high concentration in the tissues of *M. edulis*.

These results suggested that the high concentration of taurine and perhaps other amino acids may be associated in some way with the salinity of the environment. To investigate this possible relationship, *R. cuneata* was gradually moved from water of 3‰ to water of 25‰. Amino acid extracts were made from specimens at several salinities within the above range, and four individual amino acids were measured. The results of this study showed that taurine was not retained in the tissues of *R. cuneata* regardless of salinity, but that the amino acids, particularly alanine, increased with the higher saline environments.

Materials and Methods

R. cuneata was collected in the San Jacinto River, Houston, Texas. Following collection, the clams were transported to the laboratory and kept at approximately 25° C. in large aerated 50-gallon aquaria. The clams were allowed at least three days to adapt to the aquarium before being used in experimental work.

A series of five-gallon aquaria were set up in consecutive order of increasing salinities which ranged from 3‰ to 25‰. Filtered sea water was diluted to the appropriate volume with distilled water. Salinities were determined by the Mohr

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technique, as described in Standard Methods (1955). Several tests were made on *R. cuneata* to determine how rapidly they could be moved from one salinity to another. It was found that if the animals were allowed to remain in a particular tank for two days, they could be transferred without fatalities.

In order to insure correct controls for starvation a group of clams was started together in the fresh-water tank. After two days all but a dozen of the original group were moved to the next tank of higher salinity. This was repeated until all the tanks contained a dozen clams. After the final tank was reached, two days were allowed to elapse prior to making amino acid extracts from representatives of each tank. The effects of leaving clams in one tank over a period of time were checked, and up to 20 days there was no measurable change in the amino acid concentration.

The extraction and fractionation of amino acids were carried out as reported by Allen and Awapara (1960). The amino acids were analyzed by two-dimensional ascending paper partition chromatography. The amino acid extracts were spotted on Whatman filter paper #4. Solvent mixtures were 72% phenol and 65% lutidine. Both solvents were redistilled prior to use.

The quantitative estimation of alanine, aspartic acid, glutamic acid and glycine was carried out by the method of Awapara *et al.* (1950) with the following modification from Moore and Stein (1948). After localizing the amino acids on the chromatogram the spots were outlined with pencil and the paper sprayed with 1% KOH in methanol. After drying the sprayed papers in air, the spots were cut out and placed in test tubes. The tubes were put in a vacuum desiccator over concentrated sulfuric acid for three hours. Color was developed with the reagent of Moore and Stein (1948). The tubes were read at 570 mμ on a Coleman Junior Spectrophotometer.

The dry weight was determined by drying the total animal minus shell at 100° C. until the weight remained constant. The inorganic constituents (ash weight) were estimated by incinerating the dry tissues in a muffle furnace at 1000° C. The difference in dry and ash weights was expressed as per cent ash weight.

Results and Discussion

In view of the possible relationship between amino acid concentration and the hydration of tissues, the dry weight and ash weights were determined on specimens of *R. cuneata* from environments of different salinities. The results are shown in Table I.

From the dry weight studies, it is obvious that as the clams progress from a dilute to a more saline environment there is a loss of water. The ash weights, on the other hand, increase slightly in the higher salinities, reflecting an increase of inorganic constituents. These results are in agreement with earlier investigations of Fredericq (1904), Krogh (1939), and Fox (1941). These investigators demonstrated that euryhaline molluscs establish an equilibrium with their environment with respect to inorganic ions. This equilibrium is observed by a weight-volume change and a fluctuation of ion concentration depending on the salinity of the environment.

The effect of increased salinity on the amino acid concentration in *R. cuneata* is shown in Table II and Figure 1. All four amino acids increased in concentration

until the animals reached a salinity of 17 ‰. In the two higher salinities the amino acids decreased in concentration. From the data on per cent dry weights and ash weights it appears as if the loss of tissue water and gain of inorganic ions reaches a maximum in salinities of 17–20‰. This leveling off of water loss and the decrease in inorganic ion exchange in *R. cuneata* suggests a shift in osmotic control. The concurrent decrease in amino acid concentration may be associated with this phenomenon. This possible relationship between amino acids, the control of tissue water, and inorganic ions needs further investigation. These three factors may prove to be in dynamic equilibrium in euryhaline molluscs when these animals are

TABLE I

Per cent dry weights and ash weights of R. cuneata *from different salinities*

Salinity		Per cent dry wgts.	Per cent* ash wgts.
3‰	Range	18.18–20.38	2.56–4.54
	Mean	19.34	3.43
	S.D.	0.36	0.36
6‰	Range	19.21–21.40	3.54–4.43
	Mean	20.36	3.95
	S.D.	0.41	0.13
10‰	Range	21.06–24.00	3.75–4 97
	Mean	23.18	4.41
	S.D.	0.51	0.24
17‰	Range	23.54–25.76	4.31–5.97
	Mean	24.41	5.13
	S.D.	0.95	0.35
20‰	Range	25.03–26.82	5.14–6.31
	Mean	25.75	5.84
	S.D.	0.33	0.22
25‰	Range	24.62–26.86	4.63–6.12
	Mean	25.70	5.27
	S.D.	0.32	0.23

* Per cent ash weight expressed in terms of dry weight of animal.

moved from one environment to another. If such is the case, the inclusion of amino acids in osmotic control broadens considerably ideas encompassed in the process of osmoregulation.

Whether or not this increase in alanine is in response to an osmotic imbalance cannot be determined from these results. However, some studies have shown indirectly that amino acids play a role in osmoregulation. Camien *et al.* (1951) and Duchâteau *et al.* (1952) have shown that marine invertebrates have a higher concentration of free amino acids than fresh-water forms. This fact was the basis for investigating the effect of salinity change on euryhaline crabs by Duchâteau and Florkin (1955) and Shaw (1958a, 1958b, 1959). These investigations showed

that the amino acid concentration fluctuated in direct proportion with the salinity of the environment. Likewise, Duchâteau *et al.* (1961) moved the polychaet worm, *Arenicola marina*, from 100% sea water to 50% sea water, and observed a decrease in the amino acid concentration. The amino acids most affected by the change were glycine and alanine. The total decrease in amino acids was more than what would be accounted for on the basis of tissue hydration. Potts (1958) reported the effect of changing salinity on two molluscs, *Mytilus edulis* (marine) and *Anodonta cygnea* (fresh-water). The amino nitrogen decreased in *M. edulis* as this animal was moved into water of lower salinity. The reverse was true when

TABLE II

Amino acid concentration in R. cuneata *from different salinities (expressed as μmoles/gm. tissue dry weight)*

Salinity		Alanine	Glycine	Glutamic	Aspartic
3°/₀₀	Range*	3.2–9.6	3.0–6.7	3.2–6.2	1.1–3.4
	Mean	6.1	5.1	4.3	2.1
	S.D.	1.1	0.7	0.4	0.4
6°/₀₀	Range	14.3–24.3	14.5–26.4	10.1–14.7	2.3–6.0
	Mean	18.4	18.9	13.3	3.5
	S.D.	1.9	2.1	0.8	1.1
10°/₀₀	Range	82.9–108.6	29.0–38.4	19.9–22.8	5.7–9.5
	Mean	92.6	34.8	21.4	7.7
	S.D.	4.3	1.7	0.6	0.7
17°/₀₀	Range	205.7–249.2	45.9–58.2	29.5–35.8	14.5–17.4
	Mean	225.4	51.6	32.3	16.2
	S.D.	7.6	2.4	1.3	0.5
20°/₀₀	Range	179.5–209.3	26.6–36.0	21.0–29.2	8.9–12.8
	Mean	192.6	32.1	24.4	10.3
	S.D.	5.1	1.5	1.6	0.8
25°/₀₀	Range	156.5–186.7	20.8–25.6	18.3–21.6	5.0–9.0
	Mean	170.2	23.7	20.0	6.9
	S.D.	5.1	0.8	0.6	0.8

* Range represents distribution of amino acid concentrations from five separate individual animals from each salinity.

A. cygnea went into higher salinities. There is an effect of salinity on the amino acid concentration in euryhaline invertebrates. The exact relationship between amino acids, water balance and inorganic constituents needs to be worked out.

The source of alanine as well as the other measured amino acids cannot be explained readily. They may arise from the increased hydrolysis of protein. However, the high concentration of one amino acid such as alanine suggests that there may be more involved than just increased protein breakdown. A process whereby one amino acid could reach such high values could be explained by the interaction of the amino acids from protein and keto-acids from carbohydrate

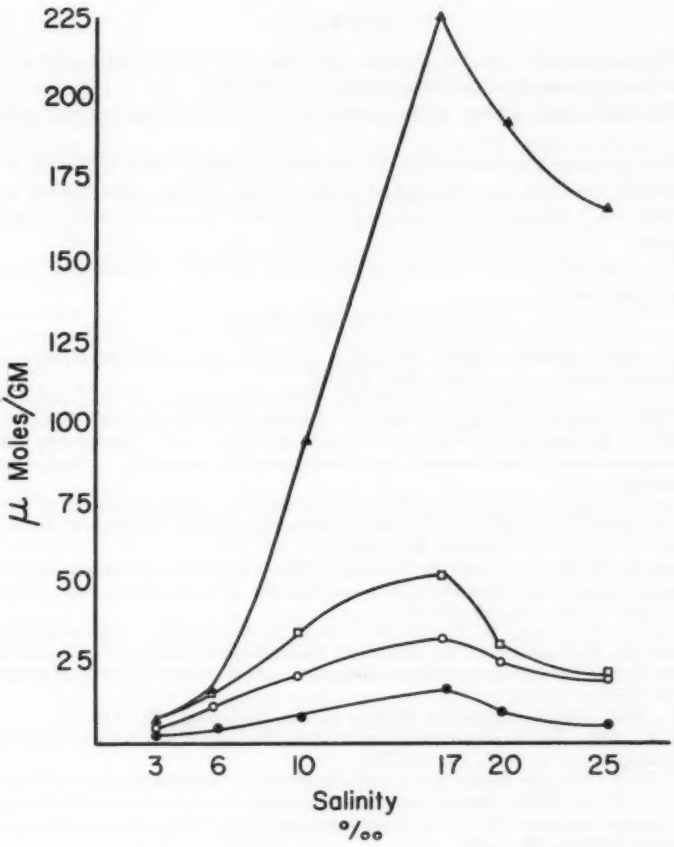

FIGURE 1. Amino acid concentration in *Rangia cuneata* taken from different salinities. Alanine ▲; aspartic acid ●; glutamic acid ○; glycine □.

metabolism via transamination. There is little evidence for this. If such were the case one might expect to observe an increase of respiration, a decrease in glycogen and an increase in one or more amino acids. Pieh (1936) and Maloeuf (1938) observed an increase in the respiration of *M. edulis* as this mussel was placed in dilute sea water. This was also true when the animals were returned to 100% sea water. Preliminary studies in this laboratory have shown a decrease in glycogen as *R. cuneata* is moved into higher salinities. These results may be interpreted as an indication of work being done in order to maintain osmotic equilibrium. At the same time carbohydrate is being used for energy purposes, the production of keto-acids provides a source of carbon chains for the formation of amino acids via transamination. Only experimental evidence will verify the latter.

SUMMARY

1. The amino acids alanine, glycine, glutamic acid and aspartic acid were quantitated in *Rangia cuneata* taken from different salinities.

2. The individual amino acids increased in concentration as the salinity increased.

3. The increased concentration of individual amino acids followed a definite pattern which was already established prior to the change into higher salinities. The pattern was: alanine > glycine > glutamic acid > aspartic acid, regardless of environment.

4. The possible role of amino acids in an animal withstanding changes in salinity is discussed.

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STUDIES ON THE ISOLATED ISLET TISSUE OF FISH. IV. IN VITRO INCORPORATION OF C^{14}- AND H^3-LABELED AMINO ACIDS INTO GOOSEFISH ISLET TISSUE PROTEINS [1, 2]

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Elucidation of the factors involved in the biosynthesis of insulin is essential for a better understanding of the etiology and development of diabetes mellitus. To this end, *in vitro* studies of the incorporation of labeled amino acids into islet cell proteins have been undertaken. In mammalian species the islet tissue is dispersed into a million or more individual islets of Langerhans, whose total mass approximates only 1% of the pancreatic mass. This makes it very difficult to separate the islet from the acinar tissue. In teleost fish, however, the islet tissue is concentrated into one or more discrete bodies called the principal islets (Diamare, 1905; Rennie, 1905); the acinar tissue, in contrast to that of mammalian species, is dispersed throughout the mesentery and located along the bile duct and within the liver. The goosefish, *Lophius piscatorius,* a marine teleost of wide distribution, was chosen because of its large and discrete aggregations of islet tissue, which are rich in extractable insulin and relatively free of exocrine tissue (Macleod, 1922). The *in vitro* study of insulin biosynthesis, using isolated islet tissue and radioactive amino acids, is based on the assumption that this process is closely analogous to the natural biosynthetic pathway. Net synthesis of specific proteins from labeled amino acids, *in vitro,* has been established for serum albumin (Peters and Anfinsen, 1950) and cytochrome *c* (Kalf *et al.,* 1959).

Previous *in vitro* studies of insulin biosynthesis using labeled amino acids and mammalian pancreas have been carried out by Pettinga and Rice (1952), Vaughan and Anfinsen (1954), and Light and Simpson (1956). Since the ratio of exocrine to islet tissue is large in mammalian pancreas, it was necessary to use large samples of tissue (weighing 10 to 100 gm.). In addition, the presence of large amounts of non-insulin proteins required the use of exacting purification procedures.

The present report deals with our studies on the *in vitro* incorporation of C^{14}-labeled amino acids into proteins using the isolated islet tissue of the goosefish. A preliminary report of these studies has been published (Bauer and Lazarow, 1961).

[1] These investigations were initiated in the summer of 1959 by the late Austin Lloyd Yates, a brilliant young graduate student at the University of Minnesota. Mr. Yates had successfully demonstrated that C^{14}-labeled amino acids, added to toadfish islet tissue *in vitro,* were incorporated into the alcohol-soluble fraction. Because of his tragic accidental death in the summer of 1959, and because all of the original protocols were lost, these studies were interrupted; they were resumed in June, 1960.

[2] This investigation was supported by research grants A-1659 and A-1887 from the National Institute of Arthritis and Metabolic Diseases, and 2G-114 from the Division of General Medical Sciences, National Institutes of Health, United States Public Health Service.

METHODS

Incubation procedure. Goosefish were obtained periodically during the summer months in the vicinity of Woods Hole, Massachusetts, and maintained in refrigerated sea water tanks. The large principal islet, located in the mesentery adjacent to the cystic duct, was removed, and the connective tissue capsule dissected away. Occasionally, the smaller secondary islet, located near the pyloric stomach, was also used. One to three pieces of islet tissue, weighing between 2 and 6 mg., were incubated at 25° C. in a micro-homogenizer tube (Fig. 1). The incubation medium

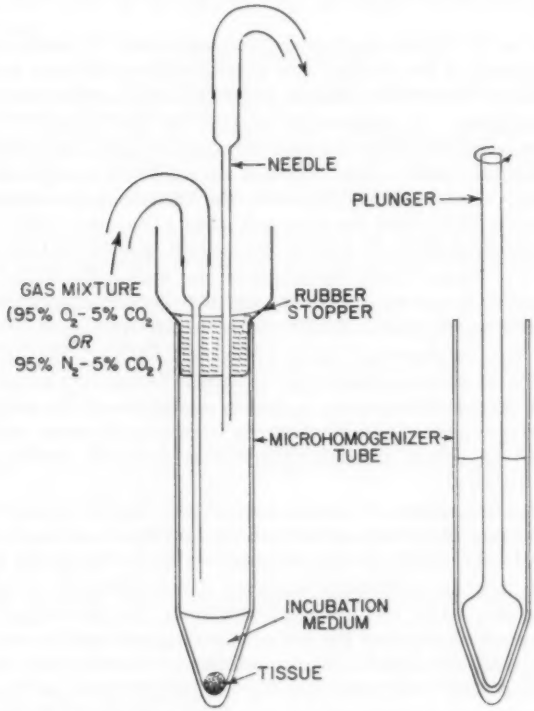

FIGURE 1. The incubation and extraction vessel.

consisted of 50 μl. of Krebs' Ringer bicarbonate buffer (Krebs and Eggleston, 1940), containing leucine-C^{14} + valine-C^{14}, or leucine-H^3 (initial pH 7.4). In experiments using C^{14}-labeled amino acids, the amino acids were dissolved in water at 11 times the desired final concentration. One part of this mixture was added to 10 parts of mammalian Krebs' Ringer bicarbonate buffer to give a final electrolyte concentration of 0.14 *M*; this is isotonic to fish blood. (Since there are 6 leucine and 5 valine residues per mole of insulin, a mixture of leucine-C^{14} and valine-C^{14} was

used in order to obtain high specific activities of the proteins synthesized.) When leucine-H^3 was used, the desired amount of amino acid was dissolved in the appropriate volume of 0.14 *M* Krebs' Ringer bicarbonate buffer.

The concentrations of amino acids used in these studies were the following: DL-leucine-1-C^{14}, 2.1 m*M*/l. (specific activity = 8.75 mC./m*M*), or 2.2 m*M*/l. (S. A. = 7.80 mC./m*M*); DL-valine-1-C^{14}, 1.6 m*M*/1. (S. A. = 10.5 mC./m*M*), or 3.0 m*M*/l. (S. A. = 6.05 mC./m*M*); DL-leucine-4,5-H^3, 0.43 m*M*/l. (S. A. = 3,570 mC./m*M*).

The micro-homogenizer tubes, containing the incubation medium, were equilibrated with the appropriate gas mixture and stoppered. They were re-equilibrated after introduction of the tissue and intermittently during the incubation period. Mixtures of 95% O_2-5%CO_2 were used in aerobic experiments, or 95% N_2-5%CO_2 in anaerobic experiments. Before introduction into the vessels, the gas mixtures were saturated with water vapor by bubbling them through a series of flasks containing water, thus minimizing evaporation of medium from the incubation tubes. The micro-homogenizer tubes were shaken periodically throughout the incubation period.

Extraction procedure. The incubation period was terminated by adding an equal volume (50 μl.) of 10% trichloroacetic acid (TCA). An additional 100 μl. of 5% TCA were added. The plunger of each micro-homogenizer was inserted and the tissue homogenized. The plunger was removed and the tubes centrifuged for 4 minutes at 3000 r.p.m. in a clinical centrifuge, and the supernatant decanted (by capillary pipette). Subsequent washings and extractions were carried out in the original micro-homogenizer tube, the plunger being used to re-suspend precipitates. The tubes were cooled in an ice bath during the extraction and purification procedures.

The precipitate was washed with 200 μl. of 5% TCA (5 times) in order to remove free amino acids. The washed residue was extracted with 200 μl. of 95% ethanol; this was repeated once, and followed by a 95% ethanol-ether (1:1), and an ether wash. The residual protein, designated as the trichlor-precipitable protein residue (TPR), was suspended in 500 μl. of acetone, and aliquots of this were immediately transferred to planchets for counting. The term "protein" includes nucleoprotein precipitated by cold TCA.

Purification procedure. The supernatant of the first alcohol extraction, designated as the first alcohol-soluble fraction (ASF), was transferred to a new micro-homogenizer, and the alcohol removed by evaporation under a stream of nitrogen. The residue was washed twice with acetone (100 μl.), twice with ether (200 μl.), and then dissolved in 100 μl. of acid alcohol (75% ethanol acidified with HCl; pH < 1.0). After centrifugation, the supernatant was removed and the residue re-extracted with 100 μl. of acid alcohol. The acid alcohol supernatants were combined and transferred to a new micro-homogenizer. Four volumes of acetone were added; following centrifugation and decantation, the precipitate was redissolved in acid alcohol and aliquots transferred to planchets for counting.

The acetone and ether supernatants obtained were pooled and plated *in toto;* this fraction is designated as the lipid solvant fraction (LSF). The acetone supernatant was also plated *in toto.* The radioactivity in this fraction represents unprecipitated proteins and soluble peptides.

Insulin-I^{131}. The I^{131}-labeled insulin used in the recovery experiments was obtained from Abbott Laboratories; it had a specific activity of 2 to 5 mC./mg.

Radioisotope counting. In the experiments using C^{14}-labeled amino acids, triplicate 25-μl. aliquots were transferred to planchets and air-dried. The samples were counted to an accuracy of 5%, using a gas flow counter (Nuclear Chicago) under conditions in which the counting efficiency was 31%. In the studies using tritium, triplicate 25-μl. aliquots were pipetted into the counting vessels; 1 ml. of hyamine and 10 ml. of toluene containing 0.4% PPO (2,5-diphenyloxazole) were added. The samples were counted to an accuracy of 5% in a Packard Tri-Carb liquid scintillation counter at an efficiency of about 4%.

Results

The distribution of radioactivity recovered in the various fractions during the washing and extraction procedures is illustrated in Table I. The counts remaining in the fifth TCA wash, presumably due to the presence of free C^{14}-amino acids, were 50 cpm/200 μl. in this experiment. This is only 3% of the counts recovered in the ASF. The counts in the ethanol-ether and ether extractions were 25 and 14 cpm/200 μl., respectively; these are less than 2% of the counts recovered in the TPR.

TABLE I

Distribution of radioactivity in various fractions isolated from goosefish islet tissue incubated 2 hours in C^{14}-labeled leucine and valine

Step	Fraction	cpm†	%*	%**
A	TCA supernatant	1,460,000	96.7	
B	First TCA wash	41,500	2.8	
C	Second TCA wash	2,290	.15	
D	Third TCA wash	192	.01	
E	Fourth TCA wash	83	<.01	
F	Fifth TCA wash	54	<.01	
G	First alcohol-soluble fraction (ASF)	1,860	.12	31.4
H	Second alcohol-soluble fraction (ASF-2)	232	.02	3.9
I	Ethanol-ether fraction	25	<.01	.42
J	Ether fraction	14	<.01	.21
K	Trichlor-precipitable protein residue (TPR)	3,790	.25	64.0

† Expressed as cpm/200 μl. (total supernatant).
* % of total counts recovered.
** % of residual counts remaining after last TCA wash.

Recovery experiments were carried out in which insulin labeled with I^{131} was added to the incubation medium, and the extraction procedure followed. The insulin-I^{131} was added to the incubation medium along with islet tissue; the C^{14}-labeled amino acids were omitted. The results, summarized in Table II, indicate that 79 to 89% of the added radioactivity was recovered in the ASF at 0 hours of incubation (*i.e.*, when TCA was added immediately after the addition of insulin-I^{131} to the incubation medium). When insulin-I^{131} was incubated with islet tissue

TABLE II

Recovery of I^{131}-labeled insulin added to islet tissue

Experiment	Incubation time (hours)	Alcohol-soluble fraction (ASF)		TCA supernatant		Trichlor-precipitable protein residue (TPR)	
		cpm	% of total	cpm	% of total	cpm	% of total
1	0	125,000	89	8,980	6.4	8,100	5.8
2a (Av. of 2 samples)	0	105,000	79	13,500	10	14,500	11
2b	1	79,400	62	26,500	21	19,600	15
2c	2	64,300	49	49,000	37	17,600	13

Each vessel contained approximately .289 μC. of I^{131}-labeled insulin and 1.44 to 1.91 mg. of goosefish islet tissue.

for one and two hours, the counts recovered in the ASF decreased to 62 and 49%, respectively. This was associated with a corresponding increase in counts recovered in the TCA-soluble radioactivity. These data suggest that the added bovine insulin is partially degraded in the presence of islet tissue.

In a similar experiment, not illustrated, the counts in both the first alcohol-soluble (ASF) and second alcohol-soluble (ASF-2) fractions were determined; 80% of the added counts were recovered in the first, and about 9% in the second.

TABLE III

Incorporation of leucine-C^{14} + valine-C^{14} into goosefish islet and liver fractions

Tissue		0-hour control				2-hour incubation				Ratio $\frac{ASF}{TPR}$
		Alcohol-soluble fraction (ASF)		Trichlor-precipitable protein residue (TPR)		Alcohol-soluble fraction (ASF)		Trichlor-precipitable protein residue (TPR)		
		No. of Samples	cpm†	No. of Samples	cpm†	No. of Samples	cpm†	No. of Samples	cpm†	
Islet	A*	10	326	10	150	22	5,900	22	7,440	0.79
	B**	2	96	2	131	3	3,860	3	6,480	0.61
Liver	A*	2	0	2	248	3	1,930	3	8,800	0.22
	B**	2	42	2	246	3	1,220	3	6,090	0.20

† The values given represent the average counts per minute/10 mg. of tissue.
* The concentration of leucine-C^{14} was 2.1 mM/l., S. A. = 8.75 mC./mM.
The concentration of valine-C^{14} was 1.6 mM/l., S. A. = 10.5 mC./mM.
** The concentration of leucine-C^{14} was 2.2 mM/l., S. A. = 7.80 mC./mM.
The concentration of valine-C^{14} was 3.0 mM/l., S. A. = 6.05 mC./mM.

TABLE IV

Effect of incubation time on leucine-C^{14} + valine-C^{14} incorporation into protein fractions of goosefish islet tissue

Time of incubation (hours	cpm/10 mg. of tissue	
	Alcohol-soluble fraction (ASF)	Trichlor-precipitable protein residue (TPR)
0	390	210
½	2,740	3,030
1	3,870	6,650
2	10,400	9,450
4	15,200	25,400
6	25,400	35,800
9	23,500	50,500
12	25,300	46,000

The concentration of leucine-C^{14} was 2.2 m*M*/l., S. A. = 7.80 mC./m*M*.
The concentration of valine-C^{14} was 3.0 m*M*/l., S. A. = 6.05 mC./m*M*.

On the basis of these recovery experiments, it was presumed that insulin present in islet tissue would likewise appear in the ASF. This assumption is consistent with the known solubility of insulin in acid alcohol (the pH of the ASF was approximately 2; this acidity is due to the presence of TCA extracted from the precipitate by alcohol). In similar experiments, where C^{14}-labeled amino acids were added to the incubation medium, the majority of the counts extracted by alcohol were likewise found in the first ASF.

A comparison of C^{14}-amino acid radioactivity incorporated into the various fractions of islet and liver is shown in Table III. The total number of counts incorporated into the ASF of islet tissue was approximately equal to that found in the TPR; the ratios of counts in the ASF/TPR were 0.79 and 0.61. By

TABLE V

Effect of aerobic and anerobic conditions on the incorporation of leucine-C^{14} + valine-C^{14} into goosefish islet tissue protein fractions

Experiment	Time of incubation (hours)	Alcohol-soluble fraction (ASF)				Trichlor-precipitable protein residue (TPR)			
		No. of samples	cpm/10 mg. tissue		% inhibition	No. of samples	cpm/10 mg. tissue		% inhibition
			Aerobic	Anaerobic			Aerobic	Anaerobic	
A*	0	2	113	113		2	383	383	
	2	3	2,900	239	91.8	2–3	4,500	1,450	67.8
B**	0	1	124	124		1	44.5	44.5	
	2	3	3,820	814	78.7	2–3	7,130	2,260	68.2

* The concentration of leucine-C^{14} was 2.1 m*M*./l., S. A. = 8.75 mC./m*M*. The concentration of valine-C^{14} was 1.6 m*M*./l., S. A. = 10.5 mC./m*M*.

** The concentration of leucine-C^{14} was 2.2 m*M*./l., S. A. = 7.80 mC./m*M*. The concentration of valine-C^{14} was 3.0 m*M*./l., S. A. = 6.05 mC./m*M*.

contrast, the total number of counts incorporated into the ASF of liver was much smaller than that in the TPR (ASF/TPR ratios of 0.20 and 0.22). Thus, islet tissue appears to incorporate a much greater fraction of the added counts into the protein component which is soluble in alcohol.

The effect of incubation time on the incorporation of amino acid radioactivity into the ASF and the TPR fractions is shown in Table IV. The counts incor-

TABLE VI

Effect of varying C^{14}-amino acid concentration on incorporation into goosefish islet fractions

Experiment	Sample	C^{14}-amino acid concentration m*M*/l.†	mg. of tissue	Alcohol-soluble fraction (ASF)		Trichlor-precipitable protein residue (TPR)	
				cpm/10 mg.	%*	cpm/10 mg.	%*
A	1	leucine 2.2 valine 3.0	5.84	4,110	100	6,380	100
	2	leucine 1.1 valine 1.5	8.58	2,220	68	4,200	71
	3		6.36	3,320		4,780	
	4	leucine 0.55 valine 0.75	5.94	3,690	101	5,160	83
	5		4.64	4,620		5,430	
B	1	leucine 2.2 valine 3.0	5.50	4,600	100	8,150	100
	2		4.99	5,480		9,450	
	3	leucine 0.22 valine 0.30	6.33	1,880	48	2,910	45
	4		5.04	2,920		5,010	
	5	leucine 0.11 valine 0.15	4.84	1,700	31	2,860	30
	6		6.56	1,340		2,380	

* Calculated as % of counts incorporated using maximum C^{14}-amino acid concentrations in each experiment (av. of duplicates).

† The specific activities were: leucine-C^{14}, 7.80 mC./m*M*; valine-C^{14}, 6.1 mC./m*M*.

porated into the ASF increased progressively with time of incubation (*i.e.*, at ½ hour, it was 2,740 cpm/10 mg. of tissue; at 2 hours, 10,400; and at 9 hours, 23,500). A similar increase with time was also observed in the TPR fraction. These results clearly indicate that the progressive increase in counts in the ASF is a result of a metabolic incorporation of the added amino acids. They also suggest that few, if any, of the contained counts are due to the adsorption of C^{14}-amino acids.

When the incubations were performed in the absence of oxygen (Table V), incorporation of radioactivity into the ASF was markedly diminished; the inhibition was 80 to 90%. Incorporation into the TPR was also inhibited in the absence of oxygen. Since the conditions of incubation and subsequent fractionation were identical, except in the substitution of 95% N_2-5% CO_2 for the oxygen mixture, these studies clearly indicate that the incorporation of amino acids is oxygen-dependent, and therefore related to an energy-utilizing process.

The original C^{14}-amino acid concentrations selected proved to be greater than that required for maximal labeling of the protein fractions. The effect of varying the concentrations of amino acids is shown in Table VI. When they were decreased to 0.55 mM/l. leucine-C^{14} + 0.75 mM/l. valine-C^{14} (*i.e.*, one-quarter of the original C^{14}-amino acid concentrations), the level of incorporation into the pro-

TABLE VII

Incorporation of amino acids into protein fractions of goosefish islet tissue: Comparison of leucine-C^{14} + valine-C^{14} of varying specific activities with leucine-H^3

Amino acid concentration (mM/l.)	Leucine-C^{14}+Valine-C^{14} 2.1		1.6		Leucine-C^{14}+Valine-C^{14} 2.2		3.0		Leucine-H^3 .43			
Sp. act. (mC/mM)	8.8		10.5		7.8		6.1		3,570			
Fraction	Alcohol-soluble fraction (ASF)		Trichlor-precipitable protein residue (TPR)		ASF		TPR		ASF		TPR	
Time of incubation (hours)	0	2	0	2	0	2	0	2	0	2	0	2
Number of samples	10	21	10	20	11	33	11	32	1	5	1	5
cpm/10 mg. tissue ± S.E.*	211 ±63	5,320 ±681	106 ±25	6,540 ±1,040	305 ±48	5,720 ±420	67 ±18	8,560 ±466	2,200	48,100 ±4,000	204	41,500 ±2,150
Ratio ASF/TPR (2-hour values)	0.81				0.67				1.2			

* Standard errors were calculated using the equation: S.E. = $\sqrt{\frac{\sigma^2}{N}}$ where σ = standard deviation.

tein fractions was essentially unchanged. When they were decreased to 0.22 mM/l. leucine-C^{14} + 0.30 mM/l. valine-C^{14} (*i.e.*, one-tenth of the original C^{14}-amino acid concentrations), the level of incorporation was decreased by only 50%.

Table VII summarizes the results obtained using mixtures of leucine-C^{14} + valine-C^{14} of varying specific activities with those obtained using leucine-H^3. The counts incorporated per 10 mg. of islet tissue were dependent upon both the specific activities and the concentrations of the amino acids used. The incorporation into the ASF, using tritiated leucine, was 7- to 10-fold greater than that obtained with C^{14}-labeled amino acids. Considering that the counting efficiency of tritium was about 4%, whereas that of C^{14} was 31%, the theoretical number of counts incorporated into the ASF using leucine-H^3 was 60 to 80 times greater than that obtained using C^{14}.

TABLE VIII

Effect of added glucose on C^{14}-amino acid incorporation into goosefish islet tissue protein fractions

Sample no.	Glucose conc. mg./100 ml.	Experiment A					Experiment B				
		mg. tissue	Alcohol-soluble fraction (ASF)		Trichlor-precipitable protein residue (TPR)		mg. tissue	ASF		TPR	
			cpm†	%*	cpm†	%*		cpm†	%*	cpm†	%*
1	0	5.13	5,580	100	14,200	100	4.26	4,600	100	9,050	100
2	12.5	3.39	6,910	124	13,800	97.2	3.79	2,860	84.0	10,600	117
3	25	4.23	5,330	95.6	11,700	82 4	4.76	2,300	50.0	5,720	63.2
4	50	5.25	4,480	80.3	6,790	47.8	4.14	1,780	38.7	4,300	47.6
5	100	4.82	3,260	58.5	3,120	22.0	4.64	1,500	32.6	3,840	42.4
6	200	3.88	2,730	49.0	5,210	36.6	4.54	1,360	29.6	3,530	39.0

The concentration of leucine-C^{14} was 2.2 mM/l., S. A. = 7.80 mc./mM.
The concentration of valine-C^{14} was 3.0 mM/l., S. A. = 6.05 mC./mM.
* Calculated as % of counts found in sample No. 1 without added glucose.
† Expressed as cpm/10 mg. of tissue.

When the counts incorporated into the ASF and TPR fractions were compared, it was observed that the ratio of counts in the ASF/TPR was greatest when tritiated leucine was used, and lowest when the valine-C^{14} concentration exceeded that of leucine-C^{14}. These results suggest that the rate of incorporation of specific amino acids into protein differs for the ASF and TPR fractions.

The effect of adding glucose to the incubation medium is shown in Table VIII. The incorporation of C^{14}-amino acids into both the ASF and TPR fractions decreased progressively with increasing glucose concentration. At a concentration of 100 mg./100 ml., the incorporation was decreased by 40 to 70% of that obtained in samples incubated without glucose.

TABLE IX

Recovery of I^{131}-labeled insulin, added to acid alcohol, followed by precipitation with acetone

Sample no.	Unlabeled insulin μg./100 μl.	Insulin fraction (acetone precipitate)		Acetone supernatant	
		cpm†	% of recovered counts	cpm†	% of recovered counts
1	100	2,420	68.0	1,150	32.3
2	50	1,620	43.9	2,080	56.4
3	20	512	13.8	3,200	86.3
4	10	315	7.8	3,700	92.4
5	5	201	5.1	3,740	95.0
6	2	149	3.9	3,680	96.3
7	1	154	4.0	3,720	96.1
8	0.5	120	3.1	3,760	96.9
9	0	266	7.5	3,290	92.6

† Expressed as cpm/10 mg. of tissue.

Purification of the ASF was accomplished by employing a method based on the solubility of insulin in acid alcohol, and its precipitation by acetone. The ASF was dried and extracted with acetone and ether in order to remove lipids. The residue was dissolved in acid alcohol, centrifuged, and the supernatant transferred to a new tube. It was then precipitated by 4 volumes of acetone, and redissolved in acid alcohol. The efficacy of this procedure was tested by adding tracer amounts of insulin-I^{131} to various concentrations of non-radioactive insulin. It was found that in the presence of 100 μg. of unlabeled insulin per 100 μl. of acid alcohol, approximately 68% of the I^{131} radioactivity was recovered by this purification procedure (Table IX). When smaller amounts of unlabeled insulin were used, a corresponding decrease in recovery was obtained.

Upon purification of the ASF, illustrated in Table X, 65 to 90% of the counts recovered were found in the purified alcohol-soluble fraction; of the total counts recovered, the lipid solvant fraction (LSF) accounted for less than 10%, whereas the acetone supernatant accounted for approximately 10%. The total number of counts recovered in these three fractions varied, in different samples; they averaged 65.2% of the counts added: incomplete recovery would be expected when small amounts of insulin were present.

The protein contents of the purified ASF and the TPR fraction were determined; these values are expressed as μg. of protein per 10 mg. of tissue. The specific activities were expressed as cpm per mg. of protein. The results are given in Table XI.

Approximately 30 μg. of protein were recovered in the purified ASF isolated from 10 mg. of islet tissue. The specific activity of the purified ASF averaged 128,000 cpm per mg. of protein, whereas the specific activity of the TPR averaged 43,000 cpm per mg. of protein. Thus, the specific activity of the purified alcohol-

TABLE X

Purification of the alcohol-soluble fraction obtained from goosefish islet tissue incubated in C^{14}-leucine + C^{14}-valine†

Sample no.	Initial cpm/ 10 mg.	Total counts recovered cpm/ 10 mg.	% recovery	Purified alcohol-soluble fraction		Lipid solvant fraction (LSF)		Acetone supernatant	
				cpm/ 10 mg.	%*	cpm/ 10 mg.	%*	cpm/ 10 mg.	%*
1	7,630	2,920	38.1	2,480	85.8	230	7.9	171	6.43
2	10,500	5,260	50.2	3,440	65.5	1,350	25.6	469	8.92
3	5,890	6,160	105	4,770	77.5	646	10.5	760	12.3
4	4,590	4,740	103	3,900	82.3	448	9.43	400	8.45
5	4,880	3,400	69.8	2,600	76.6	462	13.6	336	9.86
6	4,440	3,520	79.3	2,840	80.6	269	9.48	418	11.9
7	9,740	4,000	41.2	3,580	89.8	269	6.75	146	3.66
8	8,870	4,740	53.5	4,140	87.0	340	7.16	280	5.89
9	13,300	6,750	50.8	5,360	79.5	454	6.74	920	13.7
10	8,660	5,280	61.0	4,320	81.9	520	9.85	435	8.25

* % of total counts recovered.

† The concentration of leucine-C^{14} was 2.2 mM/l., S. A. = 7.80 mC./mM. The concentration of valine-C^{14} was 3.0 mM/l., S. A. = 6.05 mC./mM.

TABLE XI

Specific activities of the purified alcohol-soluble fraction† and trichlor-precipitable protein residue

Sample	mg. tissue	Purified alcohol-soluble fraction			Trichlor-precipitable protein residue (TPR)		
		cpm*	μg. protein / 10 mg. islet tissue	Specific activity cpm/mg. protein	cpm*	μg. protein / 10 mg. islet tissue	Specific activity cpm/mg. protein
1	3.62	7,630	26.8	93,000	9,670	—	—
2	4.40	10,500	20.5	167,000	13,000	144	39,700
3	4.06	5,890	35.4	134,000	8,670	80.0	44,000
4	5.70	4,590	29.0	135,000	8,950	152	33,600
5	4.96	4,880	33.2	78,100	4,800	119	20,000
6	4.46	4,440	34.6	82,400	5,430	113	21,400
7	5.16	9,740	29.4	121,000	10,300	—	—
8	5.38	8,870	30.9	134,000	12,700	122	56,500
9	2.92	13,300	28.0	191,000	19,900	72.3	80,400
10	3.88	8,660	30.0	145,000	10,700	79.3	52,200
Averages ± S.E.**	4.45	7,850	29.9	128,000 ±10,800	10,400	110	43,500 ±6,590

† The purified alcohol-soluble fractions used were the same as shown in Table X.

* The incorporation of amino acid into the protein fraction during a two-hour incubation period is expressed as cpm/10 mg. of islet tissue.

** The standard errors were calculated as in Table VII.

soluble fraction is approximately three times greater than that of the trichlor-precipitable protein residue.

DISCUSSION

The results obtained clearly indicate that C^{14}- and H^3-labeled amino acids, incubated *in vitro* with islet tissue, are incorporated into the protein fractions. Since added I^{131}-labeled insulin is recovered in the purified alcohol-soluble fraction, it is presumed that insulin synthesized *in vitro* by islet tissue will similarly be found in this fraction. Although the total counts incorporated into the alcohol-soluble fraction are approximately equal to that incorporated into the trichlor-precipitable protein fraction, the specific activity of the alcohol-soluble fraction is three times greater than that of the trichlor-precipitable protein residue. The incorporation of C^{14}-amino acids into the alcohol-soluble fraction is increased with increasing periods of incubation. It is markedly inhibited when the incubations are carried out in the absence of oxygen, thus suggesting that the incorporation is dependent upon an active metabolic process which requires energy. The incorporation is also dependent upon the amino acid concentration; however, maximal values are reached at leucine and valine concentrations of about 0.25 m*M*/l.

The addition of glucose decreases the counts incorporated into both the alcohol-soluble fraction and trichlor-precipitable protein residue. A similar effect of glucose on the incorporation of alanine-C^{14} into TCA-insoluble protein was observed by Sinex *et al.* (1952). Since hyperglycemia stimulates the release of insulin from the β-cell (Lazarow, 1960), it would have been expected that glucose might

increase the rate of insulin secretion. This apparent inhibitory action of glucose might be explained by the appearance of intermediates (derived from glucose) which can be used for the synthesis of insulin; this hypothesis requires further study.

The specific activities of the ASF proteins obtained in the C^{14} studies (averaging 128,000 cpm/mg. of protein) and in the tritium studies (400,000 cpm/mg. of protein) are many times greater than those previously reported using mammalian pancreas incubated *in vitro*. For example, Pettinga and Rice (1952) isolated insulin containing 1600 cpm/mg. of protein; in the studies of Light and Simpson (1956), the specific activities were less than 1000 cpm/mg. of protein.

In order to further characterize the radioactive protein found in the ASF, additional studies have been done using a specific immuno-precipitation method. Under the appropriate conditions, serum prepared from guinea pigs previously immunized to bovine insulin will form a specific insulin-antibody complex. Using the method of Skom and Talmage (1958), this soluble complex may be precipitated by reacting it with anti-guinea pig serum obtained from rabbits immunized to normal guinea pig serum. Studies carried out by Carl R. Morgan (personal communication) using insulin-I^{131} as a marker, have indicated that approximately 85% of added insulin-I^{131} (1 μg.) combines with the specific insulin-antibody and is precipitated by the anti-guinea pig serum (rabbit). Control studies, identical to the above, except for the substitution of normal guinea pig serum for the anti-insulin guinea pig serum, yielded insignificant counts in the precipitated complex.

Similar immuno-precipitation experiments have been carried out using the tritium-labeled goosefish alcohol-soluble fraction (specific activity = 400,000 cpm/mg. of protein); these strongly support the thesis that a significant fraction of the tritium label found in the alcohol-soluble fraction represents insulin which has been synthesized *in vitro* from added leucine-H^3.

The counts incorporated into the alcohol-soluble fraction of islet tissue can be expressed as mμ moles of amino acid per gm. of islet tissue per hour. In the leucine-H^3 experiment (where the amino acid concentration was 0.43 mM/l., specific activity = 3,570 mC./M, and the counting efficiency assumed to be 4%), an incorporation of 48,100 cpm/10 mg. of tissue during a two-hour period is equivalent to 7.5 mμ moles of leucine per gm. of islet tissue per hour. Since 6 of the 51 amino acid residues in insulin are leucine, the maximal rate of insulin synthesis would not be greater than 1.25 mμ moles of insulin per gm. per hour. Converting this to units per day, the maximal rate of insulin synthesis would not be greater than 4.5 units per gm. of islet tissue per day. The human pancreas contains approximately 1 gm. of islet tissue (Lazarow, 1960), and it has been reported that the totally depancreatized man can be maintained with doses of insulin as low as 20 units per day (Rockey, 1943; Goldner and Clark, 1944). It should be noted that the *in vitro* incubation studies using goosefish islet tissue were carried out at 25° C. (whereas insulin synthesis in man takes place at 37° C.) and that the rate of chemical reactions doubles with each 10° increase in temperature. Thus, the calculated maximal rate of insulin synthesis *in vitro*, based on the observed rate of amino acid incorporation into the alcohol-soluble fraction and corrected for temperature, would approximate 10 units per gm. per day; this approaches the order of magnitude of the physiologic rate of insulin synthesis by human islet tissue.

Summary

1. Goosefish islet tissue, incubated *in vitro* with C^{14}- or H^3-labeled amino acids, showed significant labeling of the protein fractions.

2. The purified alcohol-soluble fraction is presumed to contain the insulin which is synthesized *in vitro*.

3. The rate of amino acid incorporation into the alcohol-soluble fraction increases progressively with increasing time of incubation; it is decreased in the absence of oxygen.

4. The addition of glucose decreases the number of counts incorporated into the alcohol-soluble fraction.

5. In the C^{14}-amino acid incorporation experiments, the specific activity of the purified alcohol-soluble fraction (128,000 cpm/mg. protein) is three times greater than that of the trichlor-precipitable protein residue.

6. These studies support the thesis that amino acids, added to the islet tissue *in vitro*, are incorporated into insulin.

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ABSORPTION AND EXCRETION OF COPPER ION DURING SETTLEMENT AND METAMORPHOSIS OF THE BARNACLE, BALANUS AMPHITRITE NIVEUS [1]

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For hundreds of years ships have been sheathed with metallic copper or coated with copper-containing paints to prevent or minimize surface fouling. Sheathing is clearly subject to mechanical failure and once breached exposes the entire structure to attack. Copper-containing paints are effective only as long as enough toxic substance leaches from the coating to establish and maintain lethal concentrations within the barrier layer of water through which members of the fouling complex must approach the structure. These coatings gradually decrease in effectiveness during their service life, because the diffusion gradients necessary to sustained leaching are at best temporary. Millions of dollars annually and countless hours of ship time are devoted to protecting hulls from settling barnacles.

The general biology of the fouling problem, with primary emphasis upon the complex of fouling organisms, has been intensively studied in this laboratory for several years. It is the object of this communication to describe the pathways of copper absorption, circulation, and elimination by settling barnacles, and to discuss the mode of action of this toxic substance.

In a previous paper (Bernard and Lane, 1961) we have described the early stages in metamorphosis of *Balanus amphitrite niveus*. Histological localization of copper in developmental stages was determined by the dithio-oxamide (D.T.O.) method of Okamoto *et al.*, described by Gomori (1952). Specimens representing all the settling stages from normal sea water were fixed in absolute ethanol, stained for copper and then lightly stained with hematoxylin and eosin. Whole mounts, together with serial sections cut 5 and 10 microns thick in various planes of orientation, were prepared. The same procedure was repeated for specimens which had been exposed to 200 mg./kg. of copper ion in sea water for one hour.

Copper Distribution in Barnacles from Normal Sea Water

The concentration of copper ion in Gulf Stream sea water is approximately 0.005 milligrams per kilogram. *B. amphitrite niveus,* both planktonic larvae and attached forms, were collected in Biscayne Bay, where copper concentration is somewhat more variable, and were held in Millipore-filtered Gulf Stream sea water for one hour prior to histological preparation.

[1] Supported in part by a contract between the U. S. Navy Bureau of Ships and the University of Miami.

Scientific contribution No. 338 from the University of Miami, Institute of Marine Science.

A. *Planktonic cyprids*

Whole mounts of planktonic cyprids treated with D.T.O. show characteristic greenish-black precipitate of copper in the following sites (Fig. 1):

1. In ovoid to cuboidal vacuoles within the loose connective tissue underlying the postero-lateral carapace.
2. In shapeless masses in the connective tissues of the antero-ventral region.
3. In the epithelium lining the stomach.
4. In the epithelium lining the posterior two-thirds of the hind-gut.

The lateral copper-containing vacuoles of whole mounts fixed in absolute ethanol were quite consistent in pattern and number, varying from 47 to 54 in the eight specimens studied. The size ranged from 2.4–3.5 microns × 4.0–9.5

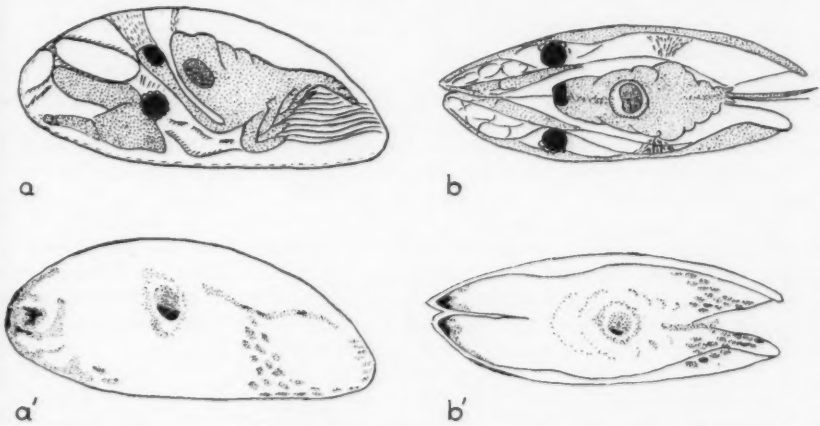

FIGURE 1. Distribution of copper ion in planktonic cyprids from normal sea water. a, a′: cyprid morphology, lateral aspect and corresponding areas of copper ion accumulation. b, b′: cyprid morphology, dorsal aspect and corresponding areas of copper ion accumulations.

microns. Copper is also concentrated within the loosely organized tissues of the anterior third of the animal, but masses of pigment granules normally present in the integument of the anterior region may mask discrete vacuolar arrangement. The outline of the stomach and of the posterior portion of the hind-gut were clearly marked by copper precipitate in whole mounts of D.T.O.-treated cyprids. A very heavy copper deposit was present in the food mass within the gut, and traces of copper-rich materials were distributed elsewhere in the gut of some animals. No copper was found in the anterior one-third of the hind-gut, in other thoracic structures, or in the digestive caeca.

While copper appears to be uniformly distributed through the stomach epithelium in whole mounts, histological sections reveal that its distribution is not uniform. The epithelial cells of the antero-ventral segment of the stomach contain more copper than cells in other areas. Granular copper deposits occur in the free

edges of the cells, distal to the nucleus and mucous secretions. In cross-section the hind-gut is lined by 5 to 8 low pyramidal cells in which copper is restricted to a semi-lunar cap next to the lumen. Copper is found only in the posterior portion of the hind-gut, although there appears to be no histological difference between these areas and the region adjacent to the stomach.

Clarke (1947) observed that barnacle larvae concentrate copper from sea water and maintain internal concentrations higher than ambient. This is a common occurrence among invertebrates utilizing hemocyanin as a respiratory pigment. Secretion of copper into the lumen of the alimentary canal of barnacle larvae in normal sea water reveals a continual turnover, suggesting that the copper ion may participate actively in the normal physiology of these animals. A functional role is confirmed by the apparent storage of copper in postero-lateral vacuoles and in the loose connective tissue at the anterior of the body.

Since copper ions are moved against the concentration gradient it is reasonable to suggest an active process requiring metabolic work by the cells of the absorbing surface.

B. *Settled cyprids*

The sites of copper concentration in this stage are essentially the same as those of the planktonic cyprid. Anterior deposits of copper disappear with the degeneration and thinning of the connective tissue of this area following attachment. Secretion of copper by the hind-gut epithelium continues even though the entire tubular digestive system is displaced by ventral rotation of the thorax in this stage. Very light deposits of copper appear within the folds of the now inactive thoracic legs. The postero-lateral vacuoles are fewer in number than in the previous stage and are concentrated at the ventral surface. Movement of the

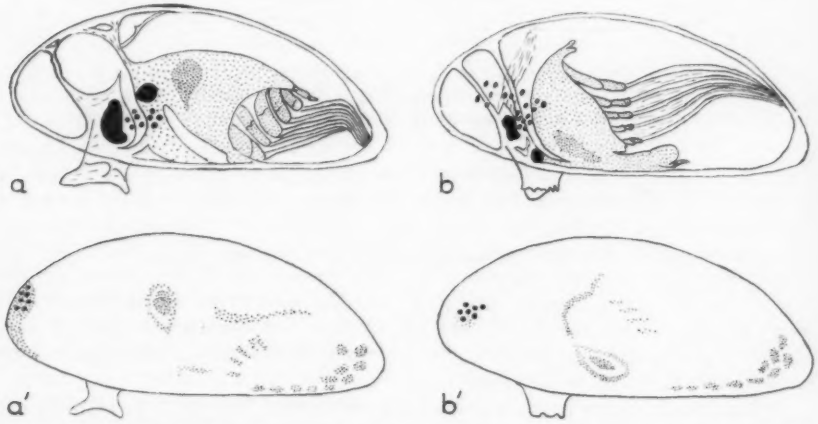

FIGURE 2. Distribution of copper ion in settled cyprid, lateral aspect. a, a′: structure shortly after attachment with corresponding sites of copper deposition. b, b′: structure just prior to decortication with corresponding sites of copper deposition.

general body tissues toward the ventral side during this stage probably accounts for this concentration (Fig. 2).

C. *Decorticated settlers*

Although a few individuals representing this stage contained small amounts of copper in isolated patches in the general parenchyma of the body, there was no consistent pattern of copper distribution.

D. and E. *Young barnacles, without and with shell plates*

No areas of copper concentration were noted, either in whole mounts or in histological sections of specimens taken during these stages.

Distribution of Copper in Barnacles Exposed to Excess Copper

Barnacles in the same metamorphic stages as above were exposed for one hour to Millipore-filtered Gulf Stream sea water to which 200 mg./kg. of cupric ion had been added. Although this concentration is 40,000 times that of normal sea water, it is yet sub-lethal. To dissolve more copper than this in sea water one must reduce the pH.

A. *Planktonic cyprids*

In spite of the great increase of available copper, the size and number of copper-containing vacuoles and the apparent concentration in connective tissues beneath the carapace are not appreciably augmented. These observations suggest either that copper reserves can only be built up over a longer period than the one-hour exposure period employed in this study, or that copper reserves are maximal in normal sea water and can not be increased further.

Deposits of copper in the epithelium of the hind-gut were much more numerous in these animals than in those from normal sea water. In barnacles from untreated sea water, copper deposits were present in approximately half the epithelial cells of the gut. The entire hind-gut epithelium, except for the portion nearest the stomach of specimens exposed to high concentrations of ambient copper, was dark with copper precipitate. Increased copper in the gut epithelium was not directly proportional to the increase in ambient copper concentration, but probably accurately reflected the increased absorption rate (Fig. 4).

In nature barnacle cyprids freely exchange copper with the medium. Established reservoirs within the body insure internal homeostasis in a medium which may fluctuate in copper content. The only sites where significant concentrations of copper normally occur are the copper-containing vacuoles and the epithelium of the hind-gut. In other areas, particularly the possible pathways from absorption sites to point of elimination, concentrations are too low to be revealed by the histochemical technique used.

When ambient copper concentrations were increased 40,000-fold, however, the amount of copper reaching the epithelium of the stomach and hind-gut appeared to exceed the excretory capacity of these cells. Copper accumulated, both in the excretory sites and in the areas of absorption. These sites of copper accumulations,

then, reveal the absorptive surfaces and sites of final elimination from the organism and suggest pathways of distribution.

The surfaces of the cyprid which are covered with a thickened cuticle, *e.g.*, legs and antennae, contained no deposits of copper. If copper were, in fact, absorbed through these regions it was immediately translocated so no local concentration occurred. The surfaces of the thorax covered by thinner cuticle, however, contained particulate copper within the epithelial cells. The flattened epidermal cells covering the posterior part of the thorax were heavily laden with uniformly distributed copper deposits, particularly within dorsal folds. The cuboidal to columnar

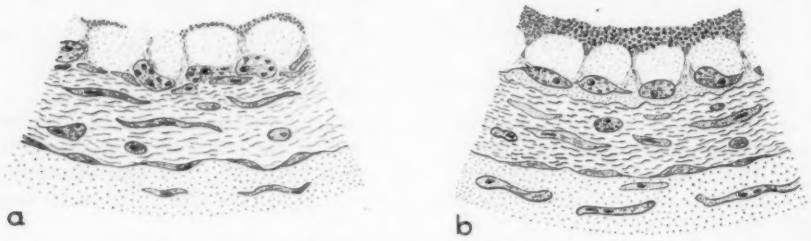

FIGURE 3. Secretion of copper ion by the gastric epithelium. (a) In cyprid from normal sea water. (b) In cyprid exposed to excess ambient copper ion.

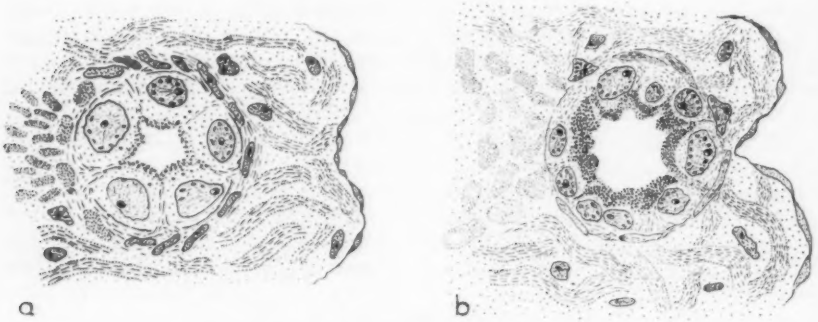

FIGURE 4. Secretion of copper ion by hind-gut epithelium. (a) In cyprid from normal sea water. (b) In cyprid exposed to excess ambient copper ion for one hour.

epidermal cells over the anterior portions of the thorax were quite heavily marked by precipitated copper in treated specimens. Thin projections of the integument with little subepidermal connective tissue, *e.g.*, mouth parts or caudal flaps near the anus, contained copper in the integument as well as in some underlying cells. Diffuse accumulations of copper were also found in the wall of the fore-gut (Fig. 5, a and b).

The internal pathways through which copper circulates from absorptive sites on certain outer surfaces to excretory regions of the gut wall were not identified

in this study. No consistent copper precipitate was observed in nerve or muscle fibers nor in the connective tissue underlying the epithelial layers. This suggests that the circulation of copper occurs in solution in blood and body fluids, where concentrations remain below detectable levels. Excretion is associated with local concentrations of copper.

Failure of the anterior third of the hind-gut to participate in copper exchange probably reflects a functional difference between this segment of the gut and all the rest of the alimentary canal (Fig. 5, c and d).

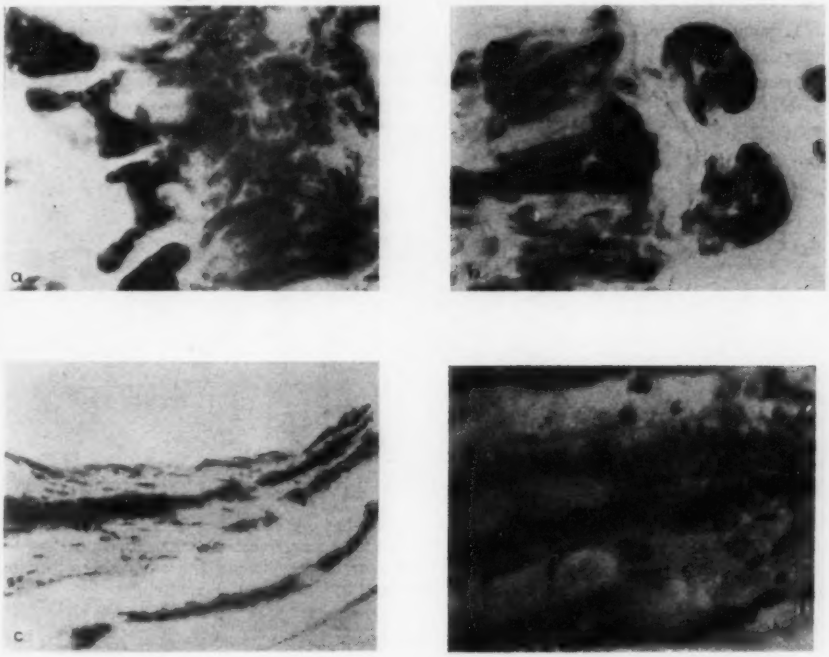

FIGURE 5. Copper precipitate in D.T.O.-treated cyprids exposed to 200 p.p.m. Cu^{++} in sea water. (a) In mouth parts and in epithelium of the fore-gut. (b) In hind-gut epithelium, anus, and anal flaps. (c) Along the posterior two-thirds of the hind-gut but not in anterior region. (d) At the point of transition between Cu^{++}-secreting and non-secreting zones.

No attempt was made to identify the mechanism of copper uptake, *i.e.*, whether it is by active ion transport or simple diffusion. This diffuse distribution of copper within absorbing cells supports the latter supposition. Active transport is suggested, however, by the polar distribution of precipitated copper within the cuboidal epidermal cells of the carapace. If the epicuticle of the carapace is impervious to ions, as is generally supposed, this copper must have been absorbed from water in the mantle cavity through the epithelium of the "mantle" and then accumulated in the epidermal cells of the carapace. The accumulations in mouth

parts and anal flaps substantiate the observation that absorbed copper moves away from the absorbing surface until some obstacle hinders its further movement.

B. *Attached cyprids*

As was described for planktonic barnacle cyprids, little increase was observed in either size or number of copper-containing vacuoles in parenchymatous connective tissue as the medium was enriched in copper ion. A trace of copper precipitate was found near the anterior pigment mass in some specimens where none was observed in animals of the same stage from normal sea water. The addition of copper ion to the medium was accompanied by a marked increase in the number and density of deposits in the hind-gut epithelium similar to that described for planktonic cyprids. Stomach secretion of copper, while increased over that

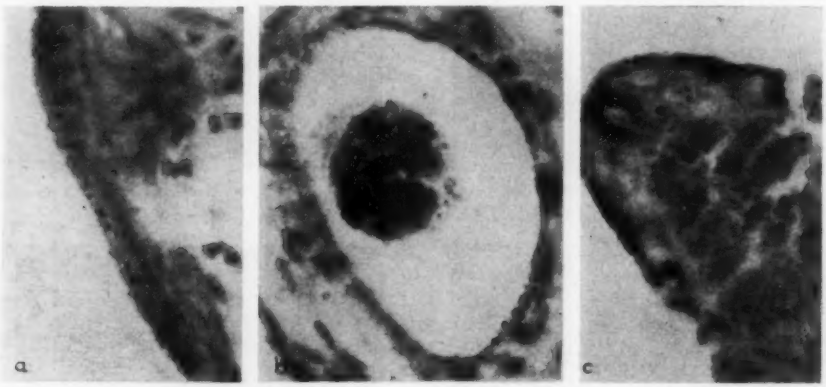

FIGURE 6. Loci of copper accumulation in attached cyprids exposed to excess ambient copper and stained with D.T.O. technique. (a) In the connective tissue underlying the cyprid carapace. (b) Within the food mass and in the secretory region of the stomach epithelium. (c) In the thoracic epidermal cells and in the secretory portion of the hind-gut.

shown by animals from this stage in normal sea water, was somewhat less than had been observed in the previous stage (Fig. 6).

If the rate of absorption of copper were proportional to its concentration within thoracic epidermal cells, then clearly less copper was taken up by cyprids after attachment than before. It is suggested that this reduced absorption results from decreased exposure of thoracic surfaces to sea water because the cirri are inactive during this stage and the mantle circulation is correspondingly reduced.

Sites of heavy copper concentration in the planktonic cyprid, such as the ventrolateral surfaces of the anterior part of the thorax, show somewhat less copper deposition in attached barnacles. The dorsal surface of the posterior half of the thorax, where the gut approaches the surface most closely, contained a fairly heavy concentration of copper (Fig. 6c). Some copper was present in the epithelium lining the fore-gut. The mouth parts and anal flaps contained considerably less copper than in the previous stage. Where precipitated copper was scant in the

planktonic stage, *e.g.,* the mid-thorax and the limb folds, none was found in the attached animals.

The distribution of copper in cells underlying the carapace resembled that already described for planktonic cyprids exposed to a high ambient copper concentration, both in the early phases where epidermal and cuticular layers are in normal contact and later when they become separated (Fig. 6a).

C. *Decorticated settlers*

Isolated particles of precipitated copper were seen in some sections of normal animals in this stage. When the copper concentration of the medium was increased to 200 ppm, there was no increase in detectable copper in the parenchymatous sheath characterizing animals in this stage. In the enriched medium, however, the cylindrical cell mass, previously described (Bernard and Lane, 1961), contained deposits of copper (Fig. 7b). These precipitates were confined to

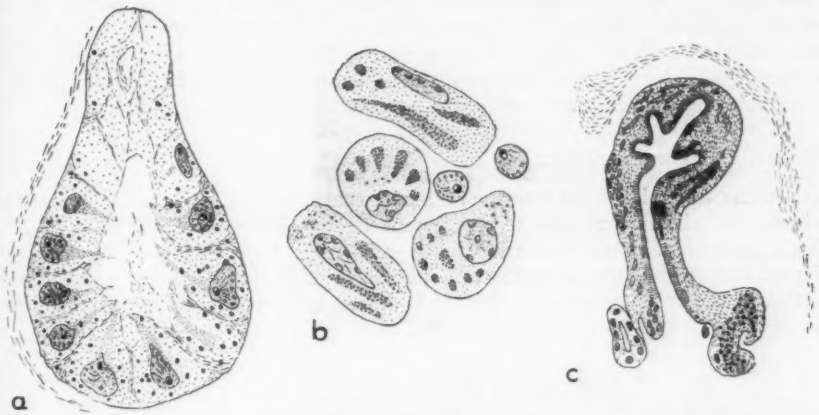

FIGURE 7. Regions of copper ion concentration in later phases of the decorticated stage by barnacles exposed to 200 p.p.m. Cu^{++} for one hour. (a) In the cytoplasm of peripheral organelle cells. (b) In fascicular vacuoles of the cylindrical cells. (c) Within one lobule of the glandular labyrinth.

fascicular inclusions within the cytoplasm of the cylindrical cells and were noted only in the latest phases of this stage when cell orientation is haphazard.

We suggested previously that inclusions within these cells are reservoirs for histolyzed material to be employed in subsequent histogenesis and organogenesis. The deposition of copper in these inclusions implies a dynamic exchange with the ambient sea water and directs attention to a possible role of these cells in concentration of other ions from sea water during morphogenesis. Vital staining experiments also indicated a rapid exchange between parenchyma vacuoles and the medium during this stage.

During the same phase at which these deposits were noted, but not in earlier specimens, some copper was detected in the cytoplasm of the cells in the peripheral

organelles. This material was perinuclear in distribution, but revealed no consistent orientation with respect to the cell surface. (See Figure 7a above.) At the same time copper secretion was observed in a few cells of a tubule connected with the large mucin-secreting organ, believed to be the primorium of the gut.

In the glandular labyrinth, a very small compound tubular gland was found to be actively secreting copper. The D.T.O.-copper precipitate appeared in the gland epithelium and in irregular blotches in the subepithelial connective tissue, as well as in the cells of a finger-like projection which marked the juncture of the gland with the main organ (see Fig. 7c).

In view of the general characters of this stage, *viz.,* no ingestive apparatus and development within a closed gelatinous sheath, it appears that copper absorption occurs only by diffusion from the medium. The lack of localized deposits in specimens from normal sea water suggests that copper ion is not essential to physiological processes characteristic of this stage. The presence of particulate deposits of copper during only one phase of development strongly suggests that free exchange with the ambient medium takes place during a restricted period of development. This period may include concentration of other ions. Activity of certain cells or cell groups, in the absence of a clear general pattern, in either secreting or absorbing copper may be a vestigial functional character from a previous stage, or may presage future activity of these cells.

D. and E. *Young barnacles*

In the transition from the decorticated larva to the young barnacle the parenchymatous sheath becomes a stiff supportive casing for the organism. The homogeneous mass of connective tissue cells and fibers is clothed by epithelial layers on inner and outer surfaces. The inner epithelium consists of squamous cells and the outer covering epithelium is at first cuboidal. The epithelial surfaces

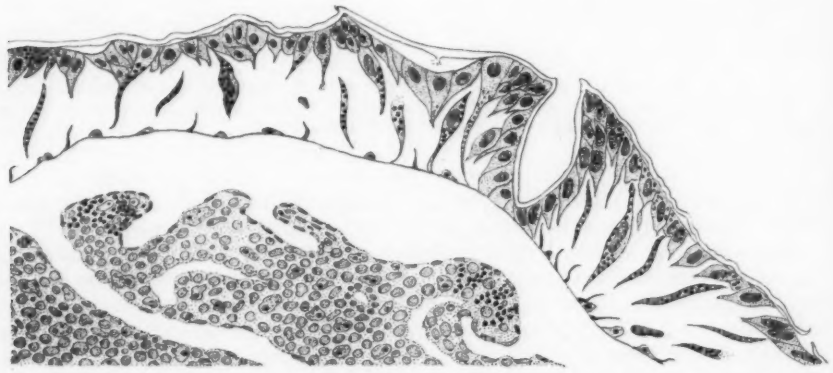

FIGURE 8. Presence of copper precipitate during the early period of shell formation in a specimen exposed to excess copper ion and treated with D.T.O. Note accumulation within the long cells penetrating the forming shell and in certain areas of the glandular region of the thorax.

are interconnected by protoplasmic extension of intermediate cells; the nuclei of these cells generally form a central layer. Within the cytoplasm of these long cells the copper precipitate was found in D.T.O.-treated specimens of the young stage before compartmental plates appeared (Fig. 8). Continued development results in a thickening of these radial cells and formation of lateral extensions from them, until a network of interlacing cytoplasmic strands unites the two epithelia. The copper deposit is no longer present after the strands have thickened. From these facts we assume that these cells are active in the absorption of solutes from sea water into the organism during this last phase of metamorphosis. It is possible that the appearance of copper in these areas at this stage is coincidental with active concentration of other ions; for example, calcium must be concentrated from sea water at this time to form the calcareous plates of the adult barnacle.

The cells in contact with the substrate form a stratified epithelium within whose lowest layer some copper is deposited.

Posterior to the stomach of the young barnacle a series of branching tubules develop which appear to be digestive caeca. In several specimens studied a copper deposit was discovered, but always in only one, or at most two, lobes of this organ. This phenomenon was noted in animals both with and without compartmental divisions.

Summary and Conclusions

1. Sites of copper ion concentration in barnacle cyprids from normal sea water, both planktonic and settled, suggest that the barnacle absorbs copper from the medium through permeable surfaces and eliminates excess copper by excretion through the epithelia of the hind-gut. Reservoirs within which copper storage may occur have been identified. A metabolic role is suggested for copper in normal physiology of barnacle settling stages.

2. Copper deposits are consistently absent from both decorticated settlers and from two stages of the young adults.

3. Exposure of planktonic cyprids and early settled forms to a high-copper medium resulted in increased rate of elimination from the gut epithelium, and identified various surface areas of the thorax as absorption sites. Transport of copper ions in solution from absorption surfaces to points of excretion is probably by way of circulating body fluids.

4. Copper deposits were observed in decorticated settlers only during later phases of this stage and in areas previously identified as active points of exchange with ambient sea water.

5. Absorption of copper from sea water containing excess copper during the stages of shell formation occurs via single-cell extensions through the thickness of the forming shell. Elimination of copper through a lobe of the digestive caecum was also noted during this period.

6. Copper localization studies on post-decortication stages suggest that copper absorption is coincident with absorption of other ions, particularly calcium, which is highly concentrated during these developmental periods.

7. Copper absorption during the cyprid-form developmental stages occurs through respiratory surfaces. It is suggested that the anti-fouling effectiveness of copper is due to interference with normal respiratory exchange.

8. The effect of the copper-ion concentration in the ambient medium on oxygen uptake of cyprids is currently being investigated and will form the substance of a subsequent communication.

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AMINO ACID SENSITIVITY OF THE DACTYL CHEMORECEPTORS OF CARCINIDES MAENAS[1]

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In contrast with the present advanced state of knowledge concerning the physiology of insect chemoreceptors the remainder of the Arthropoda are essentially unknown in this regard excepting electrophysiological observations on the xiphosuran, *Limulus* (Barber, 1956, 1961), and several fresh-water and terrestrial forms (Hodgson, 1958). Although behavioral studies have demonstrated a well developed contact chemical sense generally distributed over the integument in marine crustaceans (Luther, 1930), a physiological analysis of its sensory basis has yet to be reported. Such an analysis is likely to be of more than passing interest since, considering the diet of crustaceans, their chemoreceptors are likely to be responsive to proteins and amino acids. They may consequently be expected to present an array of relationships between molecular configuration and receptor activation unlike any which have been well studied in arthropods.

In this report we describe a simple electrophysiological preparation of crustacean chemoreceptors and present evidence concerning their responses to a number of substances, principally amino acids. A preliminary summary of this work has been given (Case, Gwilliam and Hanson, 1960).

Materials and Methods

While only first walking leg dactyls of male *Carcinides maenas* were used in these experiments, chemoreceptor activity was readily demonstrable in the leg nerves of various other decapods including *Libinia emarginata, Callinectes sapidus,* and *Paguris pollicaris.*

The dactyl was prepared for electrical recording by removing all proximal segments and leaving intact approximately a 1-cm. length of nerve consisting at this level of two prominent bundles which both possess chemoreceptor fibers. A bridge of wax was used to support the dactyl with its tip in air and the nerve immersed in sea water. Small bundles of nerve fibers were dissected and arranged on bare platinum electrodes for recording in air or mineral oil, with a recording system consisting of a Grass P5 A.C. amplifier and Tektronix 502 oscilloscope. A loudspeaker monitor was an extremely useful adjunct to photographic recording.

Chemical stimuli were applied to the dactyl as uniform-sized drops of stimulant in filtered, autoclaved sea water. Immediately before application of a test stimulus the dactyl tip was exposed to several rapidly applied drops of sea water in order to

[1] Supported by NSF Grant G5997 and NIH Grant B1890.

adapt mechanoreceptors as much as possible. During all experiments the preparation was frequently tested with *Mytilus* extract (prepared by grinding the soft parts of a *Mytilus* in about 25 ml. of sea water) or .001 to .005 *M* l-glutamic acid and discarded once responses to these fell below initial magnitudes. Ordinarily, deterioration of the preparation presented little difficulty. With reasonable care a chemoreceptor bundle remained in satisfactory condition for an hour or longer.

Responses of the dactyl chemoreceptors to test substances were evaluated by comparing the activity induced by a drop of the test substance with the effect of a drop of .001 *M* l-glutamic acid. Data were evaluated from film records and by direct aural and visual comparisons of oscilloscopic activity. For purposes of tabulation these responses were recorded on a scale of 4 in which 0 was equal to a response indistinguishable from the effect of a drop of sea water and 4 was equal to the effect of .001 *M* l-glutamic acid.

Results

1. *General*

In nearly all preparations many nerve fibers could be isolated which mediated responses to *Mytilus* extract. Except in rare instances these were difficult to isolate

Figure 1. Dactyl of first walking leg of *Carcinides maenas*.

from larger fibers carrying mechanoreceptor impulses. Indeed, there is every possibility that at least some of the chemoreceptor units themselves may respond to mechanical stimulation, although it is clear from our data that not all do. In practice, the nearly unavoidable mixing of the two types of activity was not particularly bothersome since the mechanoreceptor responses were of short latency and adapted rapidly, while the slowly adapting chemoreceptor units had considerably longer latency, ranging from 15 to 250 mscs., measured from the first mechanoreceptor spike and varying inversely with stimulant concentration.

The nature of the sensory endings mediating chemoreceptor activity in the dactyl remains unknown. Application of sufficiently localized stimuli proved so difficult that about all that can be said is that chemoreceptor activity originates from the distal one-third of the dactyl as well as from more proximal regions. While this does not eliminate a chemoreceptive role for the long mechanoreceptor hairs of the proximal two-thirds of the dactyl (Fig. 1), it makes certain that other chemoreceptors must be present. These may be the Büschelorganen of Luther (1930) which

TABLE I

Response thresholds of Carcinides dactyl chemoreceptors

Substance	Number of dactyls tested	Intensity of response	Concentration (*M*)
A. Glutamic acid and related substances:			
l-glutamic acid[1]	18	3	5.0×10^{-5}
d-glutamic acid[6]	7	1	5.0×10^{-4}
		0	5.0×10^{-5}
glycyl-l-glutamic acid[1]	6	1	4.0×10^{-4}
		0	4.0×10^{-5}
l-glutamine[2]	12	1	5.0×10^{-4}
		0	5.0×10^{-5}
n-acetyl-l-glutamic[5]	7	1	1.0×10^{-3}
		0	1.0×10^{-5}
reduced glutathione[1]	7	1	5.0×10^{-3}
		0	5.0×10^{-4}
α-methyl glutamic acid[5]	3	1	3.0×10^{-2}
		0	1.0×10^{-3}
B. Other amino acids:			
glycine[1]	6	1	1.0×10^{-4}
		0	1.0×10^{-5}
l-ornithine[5]	3	1	1.0×10^{-3}
		0	1.0×10^{-3}
d-aspartic acid[6]	6	2	1.2×10^{-2}
l-aspartic acid[1]	6	1	1.2×10^{-2}
d-leucine[6]	7	2	2.5×10^{-2}
l-leucine[6]	7	1	2.5×10^{-2}
l-proline[2]	6	2	5.0×10^{-2}
		0	2.5×10^{-2}
γ-amino butyric acid[1]	5	1	0.2
		0	0.1
C. Organic acids:			
glutaric acid[4]	8	2	3.0×10^{-2}
		0	1.0×10^{-3}
α-keto glutaric acid[1]	5	2	5.0×10^{-2}
succinic acid[3]	6	2	1.0×10^{-3}
		0	1.0×10^{-4}
acetic acid[3]	2	1	0.1
D. Miscellaneous			
sucrose[2]	3	1	0.25
lactose[2]	3	0	0.25
methanol[3]	2	1	0.10
n-propanol[3]	2	1	0.10
n-butanol[3]	3	1	0.50
sea water	2	1	150%

Sources of chemicals: [1] Nutritional Biochemicals Corp.; [2] Matheson, Coleman, and Bell; [3] Fisher Scientific Corp.; [4] Eastman; [5] Sigma Chemical Company; [6] Mann Research Laboratories; [7] Merck and Company.

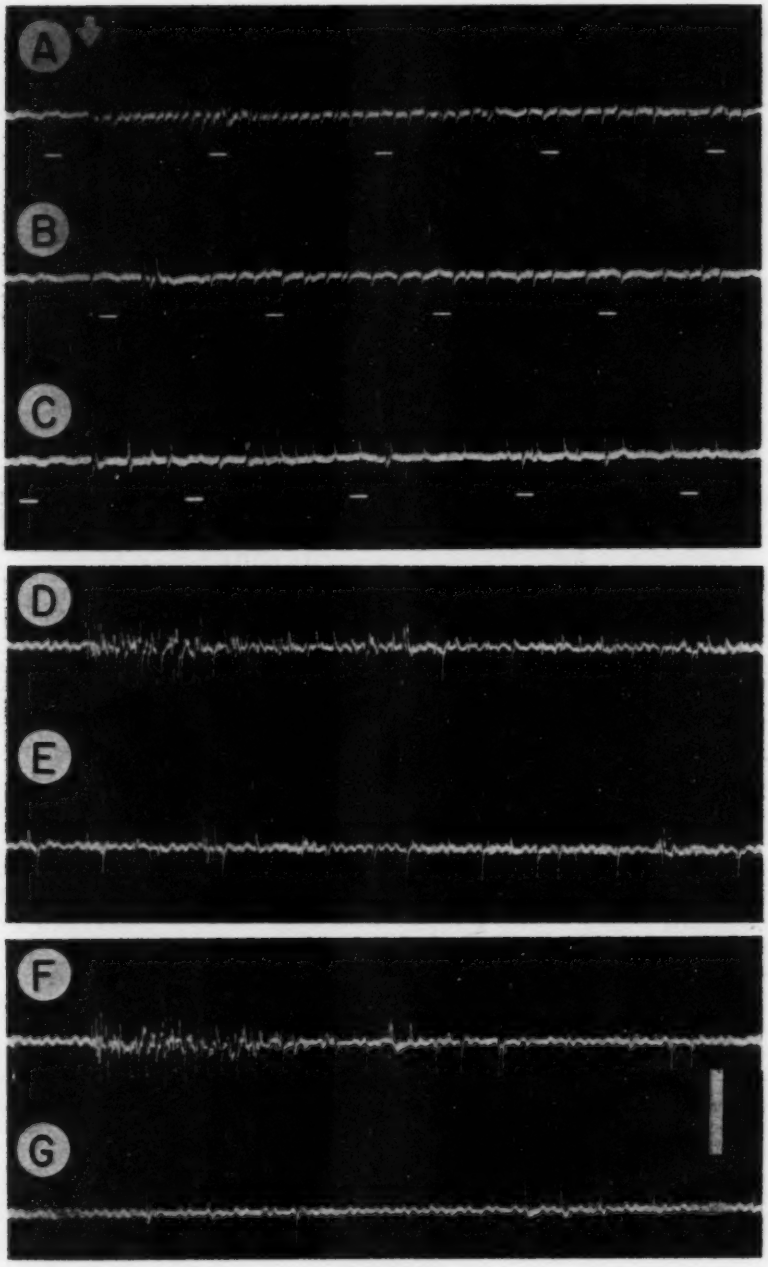

FIGURE 2.

we have also observed extending to the dactyl tip, especially in the grooves of the heavily chitinized cap. Both these and the mechanoreceptor hairs have been shown to be permeable to aqueous solutions of crystal violet in preparations for which we are indebted to Dr. E. H. Slifer, who finds the presence of such permeability in insects to be characteristic of chemoreceptive endings (Slifer, 1960).

2. Receptor specificity

In considering these data it must be remembered that our experimental technique has confined the study to a selected category, namely those sensory units which are responsive both to *Mytilus* extract and dilute glutamic acid. It may therefore be presumed that these observations on specificity are not applicable to the total chemoreceptor population of the dactyl.

As shown in Table I, *Carcinides* dactyl chemoreceptors have limited sensitivity to many stimuli, which include various alcohols, sugars, acids, amino acids, and hypertonic sea water. They are moderately sensitive to a few amino acids and exhibit marked sensitivity only to l-glutamic acid among the substances tested. Although these receptors are somewhat responsive to acidity, that property of l-glutamic acid cannot be the primary source of its stimulatory ability since the dilution at effective concentrations is so great that there is a negligible pH change in sea water. At higher concentrations the receptors do not distinguish between neutralized and acid glutamic solutions. Further strong evidence of a high order of specificity in the glutamic response is the fact that all derivatives of glutamic acid which were available to us were no more active than glycine.

Of the three sets of optical isomers tested, those of glutamic acid and leucine were distinguished by preparations of only one to three units and all three by preparations containing many active units. In some instances (Fig. 2, A, B, C) the optical isomers of glutamic acid activate different units. We were unable to detect differences in the responses of single units to d- and l-aspartic acid although this does not preclude the existence of such differences because only a few such preparations have been tested. The characteristic responses to d- and l-leucine are illustrated in Figure 2, D–G, where d-leucine produces a considerably more prolonged burst than its antipode, although the initial phases of the responses to the two isomers are nearly identical in this instance.

In the light of the pronounced specificity which the dactyl chemoreceptors have been shown to have towards glutamic acid it becomes of interest to inquire into the nature of the active element of *Mytilus* extract. Amino acids are almost certainly involved since the extract does not lose potency upon boiling for five minutes and is dialyzable. Moreover, the same receptor population seems to be involved in the response to glutamic acid and to *Mytilus* extract since adapting the dactyl to either abolishes the response to the other.

FIGURE 2. Responses of *Carcinides* dactyl receptors to chemical stimulation. A, 5×10^{-3} *M* l-glutamic acid; B, 6×10^{-4} *M* l-glutamic acid; C, 3×10^{-2} *M* d-glutamic acid; D, 2.5×10^{-2} *M* d-leucine; E, same as D after elapse of 0.5 second; F, 2.5×10^{-2} *M* l-leucine; G, same as F after elapse of 0.5 second. A–C and D–G are from the same preparations. In A–C a larger mechanoreceptor unit is active and indicated by arrow to show chemoreceptor latency. Time base 100 mscs. between markers in A–C and the same throughout. Bar at lower right, 50 μV.

3. *Behavioral correlations*

In confirmation of Luther's observations, blinded *Carcinides* oriented readily to bits of mussel placed near any limb. Before the animal made the orienting movement, the nearest dactyl frequently probed the bit of tissue previous to making a precisely executed turn to present the tissue to the mouthparts. Inert materials were ignored. When *Mytilus* extract or .001 *M* l-glutamic acid was applied to the walking legs of crabs restrained on their backs in water shallow enough to expose all limbs, thus assuring stimulus localization, no sign of recognition could be seen. However, under the same conditions application of either substance to the chelae caused the beginning of feeding movements even before the stimulated claw was touched to the mouthparts. Touching the chelae of an unrestrained crab with either substance was followed by movement of the chelae to the mouth and chewing without orienting movements of the whole animal.

Discussion

The dactyl chemoreceptors of *Carcinides* are considerably more sensitive to amino acids than any arthropod preparation so far described and, indeed, approach in sensitivity the remarkable performance of insects responding behaviorally to sugars (see tabulation in Dethier and Chadwick, 1948, p. 244). Hence, when one considers that feeding responses were induced in *Carcinides* with pure solutions of glutamic acid, together with the high concentrations in animal tissues of glutamic acid, both free and bound (Meister, 1957), it becomes clearly possible that effective localization of food can be made simply on the basis of detection of low concentrations of amino acids.

The poor response of the dactyl preparation to reduced glutathione, known to be a highly specific inductor of feeding responses in at least two coelenterates (Loomis, 1955; Lenhoff and Schneiderman, 1959), reduces the possibility that there is a feeding-inducing substance common to large groups of animals, a suggestion which receives further support from the fact that the major feeding stimulus in the mosquito, *Culex pipiens,* is yet another compound, adenylic acid (Hosoi, 1959). It would therefore seem unwise to venture generalities concerning feeding-inducing substances except that they are likely to be of low molecular weight.

Since the observations of Piutti (1886) the optical isomers of a number of amino acids have been known to produce different tastes in man. The natural isomers may be tasteless, "meaty" in the case of glutamic acid, or bland, while d-amino acids commonly are sweet (Berg, 1953). Occasionally an isomer falls in one human taste modality and its antipode in another: l-leucine and l-isoleucine are bitter and their antipodes are sweet. These relationships, involving radical changes in taste upon subtle structural modification, have never been subjected to neurophysiological investigation despite the long time they have been known. The present findings appear to be the first to extend these observations to organisms other than man and are particularly significant in that they show at least some optical isomers of amino acids are distinguishable strictly in terms of the activity of small chemoreceptor populations. Despite the limited nature of this survey it is already apparent that at least three means exist by which these isomers can elicit differing receptor activity: they may simply activate different receptors, or have different threshold concentrations or adaptation times for the same receptors.

The molecular basis of these possible modes of action obviously cannot be discussed in the light of available direct experimental evidence. However, it is interesting to recall that metabolic systems acting upon d-amino acids are quite rare in multicellular organisms. To mention a pertinent example, active transport of amino acids across the mammalian intestine is specific to the l-isomers (Fridhandler and Quastel, 1955). Such observations lend support to various commonly invoked receptor activation schemes in which stimulant-receptor complexes are envisaged as involving formation on the basis of van der Waal's forces (see discussion by Dethier, 1956). But should more instances appear in which d-amino acids have more long lasting effects than their l-isomers, as appears to be the situation with d- and l-leucine, it may become necessary to consider the possibility of chemical processes functioning in receptor clearance, even though the behavior of the insect sugar receptor argues to the contrary.

Summary

1. Dactyl chemoreceptors were electrophysiologically demonstrated in *Carcinides maenas, Libinia emarginata, Callinectes sapidus,* and *Paguris pollicaris.*

2. In *Carcinides* the receptors respond to a boiled dialysate of *Mytilus,* and to l-glumatic acid at dilutions in excess of 5.0×10^{-5} *M.* Derivatives of glutamic acid and other amino acids are less stimulatory.

3. Optical isomers of glutamic acid, aspartic acid and leucine are distinguished by small populations of *Carcinides* dactyl receptors.

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BEHAVIORAL ASPECTS OF PROTEIN INGESTION BY THE BLOWFLY PHORMIA REGINA MEIGEN[1]

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The problem of specific hungers correlated with unique metabolic requirements has been studied extensively in the vertebrates. The greatly augmented sodium chloride ingestion of persons suffering from adrenal insufficiency is a classical example. Aside from other pathological examples, the most widely studied normal instance of specific hungers is that associated with pregnancy.

It is not altogether surprising that deviations from a standard maintenance diet should occur at times of metabolic stress. Specific hungers have long been known to occur among insects during periods of egg development. Many mosquitoes require a blood meal before they can bring their eggs to ripeness. Similarly, many flies need a meal of protein for oviposition.

While much attention has been directed toward the developmental and hormonal aspects of this phenomenon, there have been few investigations into the behavioral background, specifically in answer to such question as: does the insect actually seek sources of protein? does it ingest protein preferentially? Pospíšil (1958) studied the olfactory responses of a number of saprophilic flies to such odors as skatol, which is associated with decaying proteinaceous material. He found that the fly, during the period of egg development, responded positively to skatol even when it had fed to repletion on carbohydrate; at other times the replete fly was unresponsive. Strangways-Dixon (1959, 1961) reported that the ratio of protein and carbohydrate ingestion by *Calliphora erythrocephala* Meigen varied during the reproductive cycle and that protein was ingested in relatively large quantities during early stages of egg growth as compared to low ingestion during the period of yolk formation. Carbohydrate ingestion followed an inverse course.

The studies reported in this paper were directed toward an understanding of the changes in feeding behavior during the reproductive cycle of the fly *Phormia regina* Meigen and the mechanisms underlying them.

Materials and Methods

Three types of feeding experiments were undertaken. First, flies were placed in individual cylindrical cages constructed of Nylon mesh. Each cage measured 2 cm. in diameter and 5 cm. in length. Through the floor of the cage projected the tips of two 5-ml. volumetric pipettes that had been bent into the shape of a J. The pipettes, which were supported by a clamp and base, served as support for the cage. Every 24 hours the volume of fluid ingested from each pipette was measured by refilling with a hypodermic syringe to the original fluid level and then reading the

[1] This work was aided by Grant G-6015 from the National Science Foundation.

volume directly from the syringe. Evaporation controls were run concurrently (Dethier and Rhoades, 1954).

Second, flies which had been fastened to sticks according to the technique of Dethier and Chadwick (1947) were fed to repletion, and the duration of feeding recorded. Since under given conditions there is a constant relation between the duration of feeding and the volume of fluid imbibed (Dethier, Evans and Rhoades, 1956), the amount of fluid taken could be calculated when desired after sample crops had been removed and weighed for calibration.

Third, flies from which the wings had been removed were allowed to run free on horizontal surfaces where they would encounter lines of fluid drawn in concentric circles around them.

Finally, ingestion was studied by the above-mentioned techniques in flies from which either the ovaries, corpus allatum, or median neurosecretory cells had been removed. Surgical procedures when used were those described by Thomsen (1952) and Dethier and Bodenstein (1958).

In addition to studies of ingestion, preliminary electrophysiological measurements of receptor activity were made by recording through the side wall of the chemoreceptive hairs after the method of Morita (1959). The equipment and techniques were those of Wolbarsht and Dethier (1958).

The flies were from a culture maintained in the laboratory since 1947. Originally homogenized liver was the protein employed for testing. Since similar results could be obtained from a 10% solution of Difco Brain-Heart Infusion, this was eventually substituted in all experiments. It contained, in addition to infusions of calf brains and beef hearts, 2.5 g. disodium phosphate, 5 g. sodium chloride, 2.5 g. bactodextrose, and 10 g. proteose peptone per liter of dry material. For some electrophysiological tests a crystalline bovine hemoglobin was employed. Sucrose was the carbohydrate employed.

Results

Patterns of protein ingestion

Since flies were not able to survive longer than a maximum of four days on a diet of protein alone (*e.g.*, liver homogenate, brain-heart extract), their feeding behavior over long periods could be studied only by providing carbohydrate. It was decided to provide separate sources of protein and carbohydrate concurrently rather than to mix the two. The intake of unadulterated protein could thus be studied.

Under these circumstances the volume of protein consumed daily was found to vary markedly over the lifetime of the fly. There was, furthermore, a difference between the feeding patterns of the sexes. Males, whether mated or not, gradually increased their intake from the time of emergence until the fourth to eighth day (Fig. 1). Thereafter the value reached a low level which was sustained until death. The pattern was similar for virgin females (Fig. 2), but the amount of protein consumed was greater. Mated females, on the other hand, laid eggs sometime between the tenth and fifteenth day, and within 24 hours again increased their protein consumption (Fig. 3). The quantity consumed daily by flies that had not laid eggs was always greater than that consumed by flies at any period after they had laid their first batch of eggs.

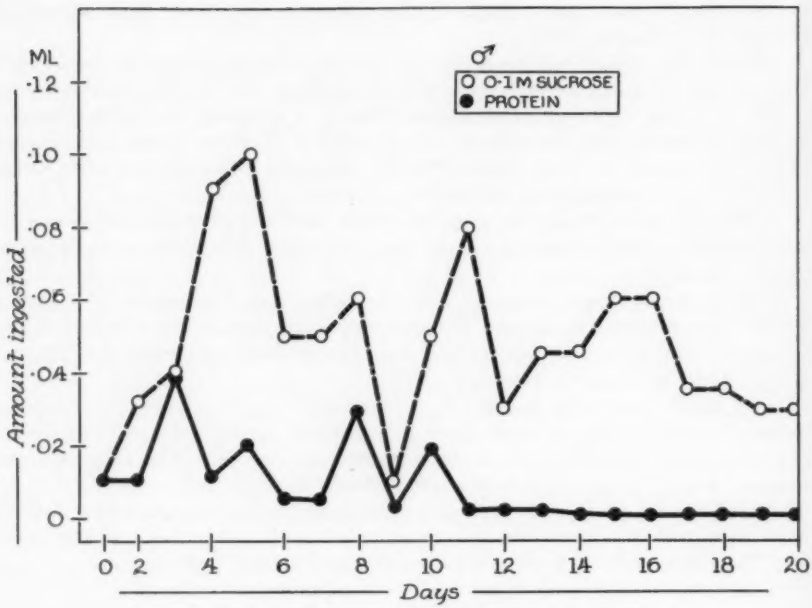

FIGURE 1. Daily intake of 0.1 *M* sucrose and brain-heart extract by a male blowfly in a two-choice situation.

Patterns of carbohydrate ingestion

When carbohydrate (0.1 *M* sucrose) was the only material offered to flies, the pattern of daily intake over the lifetime of the fly was the same for both sexes. Little was taken on the first two days after emergence. Thereafter the intake reached a high value and gradually declined until death some 50 days later for the longest-lived males and 60 days later for the longest-lived females. During this period the daily intake exhibited marked fluctuations, but these were related to differences in activity correlated with variations in the climate of the laboratory. The experiments were conducted under constant lighting but not under constant temperature, humidity, and barometric pressure.

Preferences

If the flies had free access to both protein and carbohydrate at all times from the day of emergence, carbohydrate was nearly always taken in greater volume than protein. If, however, the flies were denied access to protein and maintained on a minimal (0.0001 *M*) carbohydrate diet for the first five days of adult life, the subsequent pattern of carbohydrate and protein intake was quite different. In the case of the males, protein intake remained very low, as before, but carbohydrate intake was very high the first two days (Fig. 4). In the case of virgin females protein

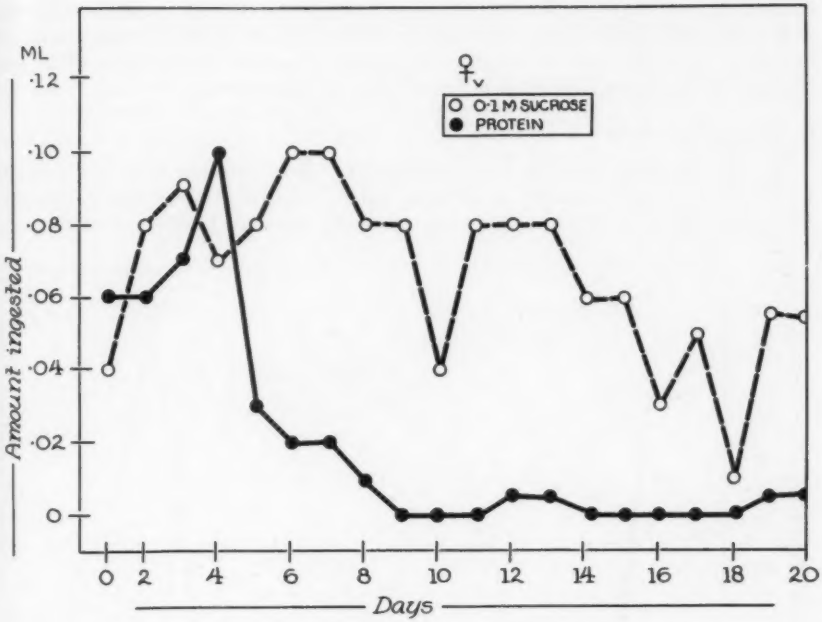

FIGURE 2. Daily intake of 0.1 *M* sucrose and brain-heart extract by a virgin female blowfly in a two-choice situation.

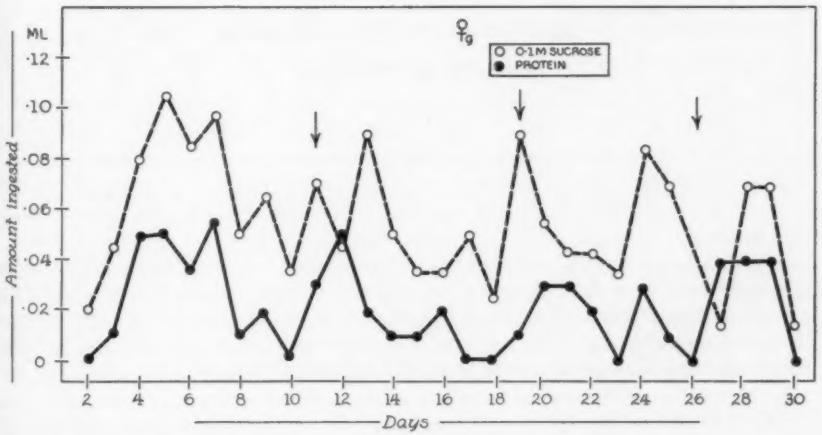

FIGURE 3. Daily intake of 0.1 *M* sucrose and brain-heart extract by a gravid female blowfly in a two-choice situation. Arrow indicate days on which eggs were laid.

intake was very high the first two days and exceeded carbohydrate intake (Fig. 5). If females were denied protein for 10 days, the subsequent preference was even more marked. After 20 days of protein deprivation, a protein preference still existed but was no more pronounced than it had been on the tenth day.

Concentration effects

In the foregoing tests the concentration of sucrose selected (0.1 *M*) was that which flies normally consume in greatest volume over a 24-hour period (Dethier, Evans and Rhoades, 1956). It is not, however, the most highly stimulating con-

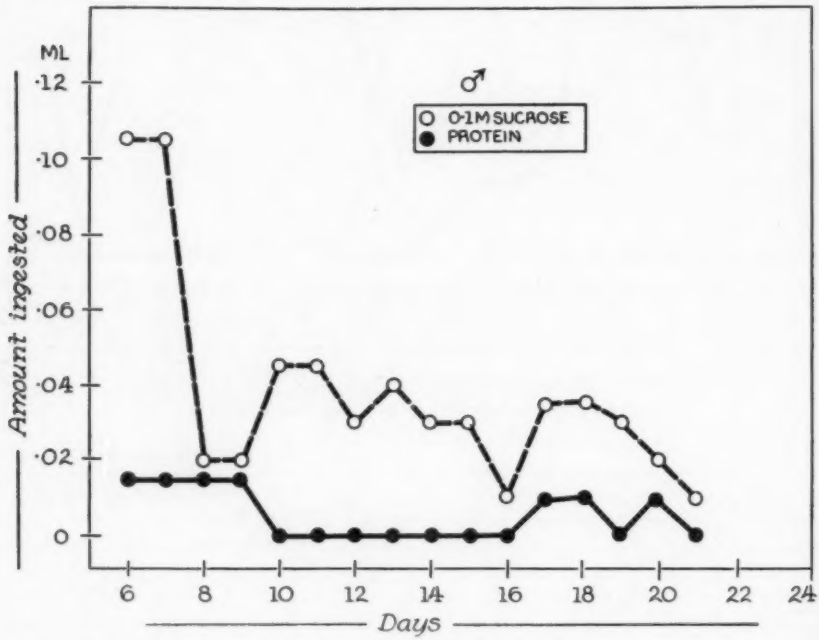

FIGURE 4. Daily intake of 0.1 *M* sucrose and brain-heart extract by a male blowfly in a two-choice situation when it has been deprived of protein for five days.

centration insofar as the sense organs are concerned. Consequently, other series of comparisons were made between protein and various concentrations of sucrose. It is clear from Figure 6 that the availability of a highly stimulating sugar greatly reduced the intake of protein. Conversely, when only a weakly stimulating sugar was available, protein intake was markedly enhanced (Fig. 7).

Initial responses

While the volume consumed has a certain validity as a test of preference, a much clearer insight into the behavior of the fly is obtained by observing its reactions

immediately upon presentation of the different solutions. For this purpose a fly was placed on a sheet of non-absorbent hydrophobic paper and three concentric rings of solution were drawn around it with a camel's-hair brush. In one set of tests the rings from center to outside were in the order water, protein, sucrose; in another set, water, sucrose, protein. Three kinds of flies were tested under these conditions: five-day-old males, starved 24 hours, and previously maintained on 0.1 *M* sucrose; five-day-old virgin females with a similar history; five-day-old virgin females 24 hours starved and previously maintained on protein solution.

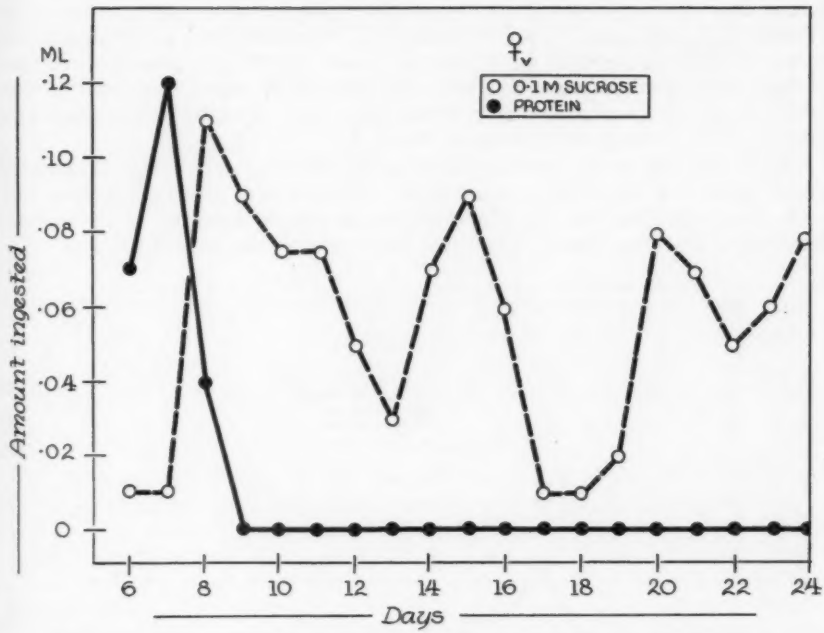

FIGURE 5. Daily intake of 0.1 *M* sucrose and brain-heart extract by a virgin female blowfly in a two-choice situation when it has been deprived of protein for five days.

All flies upon encountering water stopped and drank to repletion; thereupon the water ring became a barrier. Each time a leg encountered the ring the fly turned away where it had previously turned toward the water. This change in response was in itself interesting because it suggested that a solution that was initially acceptable had truly become repellent. Tests in which the water had been absorbed into the paper (thus presenting no unbroken surface) showed, however, that flies after sucking to repletion merely walked across the damp area instead of being repelled. The differences in behavior in the two cases may be explained if we assume that the ring of water stimulated two sets of receptors, the water receptors and mechanoreceptors (the effect of surface tension), the former mediating acceptance, the latter,

rejection. In the thirsty fly the acceptable stimulus overrides the unacceptable one. This balancing of antagonistic stimuli acting on the tarsi and the change in effectiveness with change in internal state is well known (Dethier, 1955; Dethier and Evans, 1961).

Having drunk water, the flies turned away from it. Upon each new encounter they avoided it. After a few minutes, however, their behavior changed. They now waded through and continued until they encountered the next ring of solution. When six-day-old females which had been maintained since emergence on a protein-free diet encountered the protein ring first, they drank the solution avidly, then turned away from it, then followed one of three patterns upon encountering 0.1 *M* sucrose: (1) drank some 0.1 *M*, then ignored it, but would drink 1.0 *M* sucrose if it was presented; (2) ignored 0.1 *M* sucrose, drank 1.0 *M*; (3) ignored all sucrose. If they encountered the sugar ring first, they fed fully on sugar, then drank protein while repeatedly ignoring sucrose. If, however, after drinking protein they were offered 1.0 *M* sucrose, they invariably drank it.

Males maintained on a sucrose diet alone and females which had had protein 24 hours before the test tended to act alike. Encountering protein first they took little or none of it but then drank considerable amounts of sucrose. If they encountered sugar first, they drank a great deal and then virtually ignored protein.

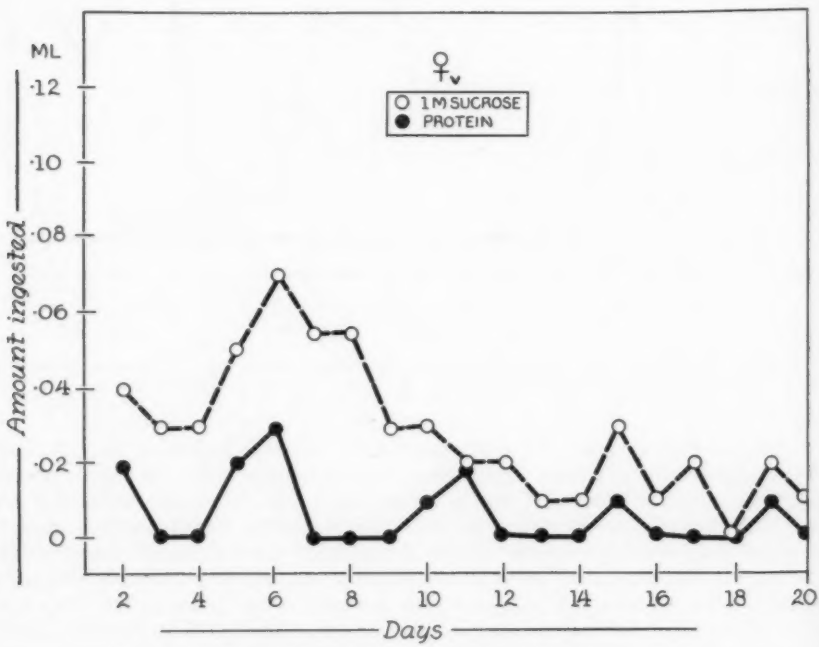

FIGURE 6. Daily intake of 1.0 *M* sucrose and brain-heart extract by a virgin female blowfly in a two-choice situation.

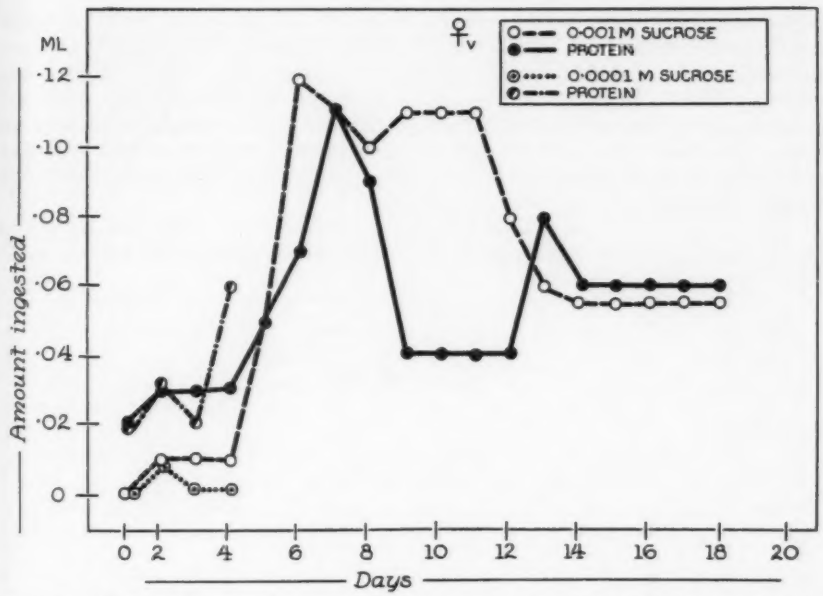

FIGURE 7. Daily intake of sucrose and brain-heart extract by a virgin female blowfly when the protein was paired with 0.001 *M* sucrose and when it was paired with 0.0001 *M* sucrose.

Number and duration of visits in a two-choice situation

In order to assist in correlating the behavior of flies taking single drinks with the measurements of total daily intake, records were made of the number and duration of visits made by flies in the two-choice situation. The records showed that females which had been denied protein made approximately equal numbers of visits to each of the two pipettes but that they took only small nips of the carbohydrate and long draughts of the protein.

Males and females which had had free access to protein also made equal visits to both pipettes but took very few drinks of protein. Drinks of sugar were more hearty.

Anosmic flies

Since all of the protein solutions employed possessed distinct odors, an attempt was made to assess the role of odor by measuring intake and observing the reactions of flies that had been rendered anosmic by removal of the antennae, labellum, and labial palpi. Anosmic flies were tested in all of the situations already described. In no case did their behavior differ from that of the normal flies.

Contact chemoreceptors

It was clear from observing the behavior of flies in the ring tests that they were able to differentiate between protein and sucrose by means of the tarsi and the

labellum. Accordingly, tests were made on the chemosensory hairs of these appendages. Small drops of either protein or 0.1 *M* sucrose were applied to tarsal and labellar hairs of protein-hungry females and the presence or absence of proboscis extension noted. In each case care was taken first to satiate the fly with water and to test each hair first with water. About 40 different labellar and 20 different tarsal hairs were tested. The tests showed that some hairs were sensitive both to protein and to sugar; others, to sugar only. At this time no hairs were found which were sensitive to protein but insensitive to sugar.

Preliminary electrophysiological findings are in accord with these behavioral results, but the matter requires more extensive investigation before the activity of

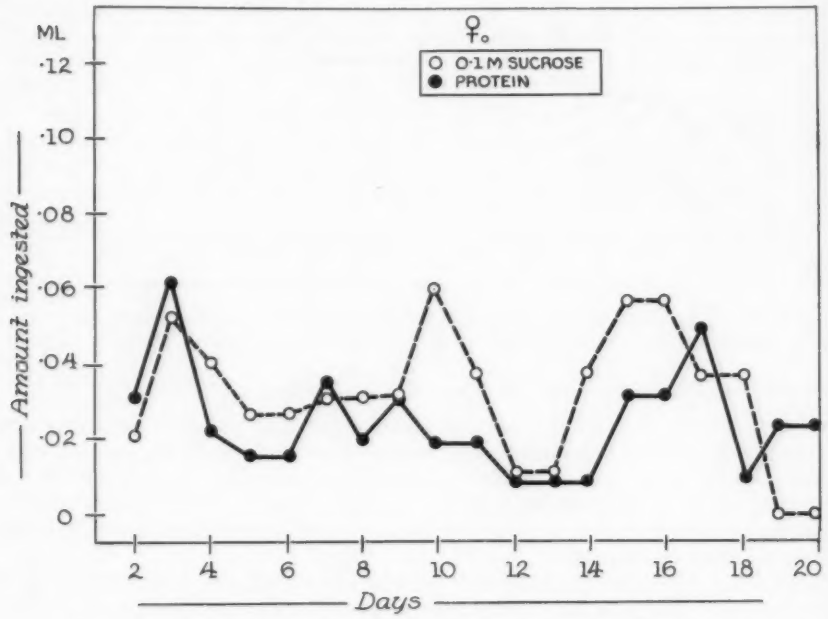

FIGURE 8. Daily intake of 0.1 *M* sucrose and brain-heart extract by a virgin female blowfly from which the ovaries have been removed.

each of the several neurons in the hair is understood. One of the long (ca. 300 μ) marginal labellar hairs tested was sensitive to water, fructose, NaCl, brain-heart extract, and crystalline hemoglobin. Activity was detected in three fibers (Fig. 10). One fiber, as Mellon and Evans (1961) have shown for these hairs, responded to water, one fiber to sugar, and one to sodium chloride. When sugar was applied, both the water fiber and the sugar fiber responded (Fig. 10B). When 1 *M* sodium chloride was applied, the water fiber was suppressed and only the salt fiber responded (Fig. 10D). When a mixture of fructose and sodium chloride was applied, three fibers (water, sugar, and salt) responded (Fig. 10G). When brain-

TABLE I

Total volumes (ml.) of protein and of sucrose taken in the first eight and first 22 days of adult life

Sex and condition	Protein		Sucrose		Total fluid
	8 days	22 days	8 days	22 days	22 days
Virgin female	0.25	0.27	1.08	1.33	1.60
Gravid female	0.26	0.33	1.03	1.37	1.70
Ovariectomized female	0.12	0.19	0.82	1.01	1.20
Female with sham operation	0.17	0.26	0.98	1.10	1.36
Male	0.07	0.09	1.05	1.14	1.23
*Allatectomized female	.12	.29	.25	.84	1.13
*Normal female	.16	.20	.19	.81	1.00
*Female with sham operation	.18	.27	.22	.70	0.97

* Flies in these categories were held for six days before testing. Tests on all other flies began on the day after emergence.

heart extract was added response could be detected in two fibers only (Fig. 10C), but it is not clear at this time which two fibers are responding. In an attempt to clarify this point a mixture of brain-heart extract and fructose was tested and also a mixture of brain-heart extract and sodium chloride. In each case activity could

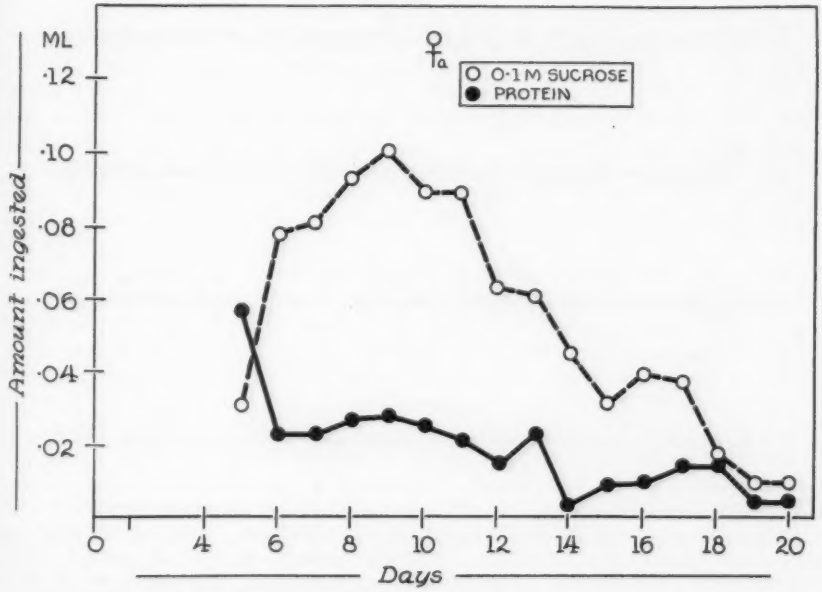

FIGURE 9. Daily intake of 0.1 *M* sucrose and brain-heart extract by a virgin female blowfly from which the corpus allatum has been removed.

be detected in two fibers only (Fig. 10E and F). Crystalline hemoglobin appeared to stimulate only one fiber.

Tests with a medium-sized hair (ca. 100 μ) presented a different picture. This hair responded to water, sugar, and salt; however, when either brain-heart extract or crystalline hemoglobin was applied, all electrical activity was reversibly blocked.

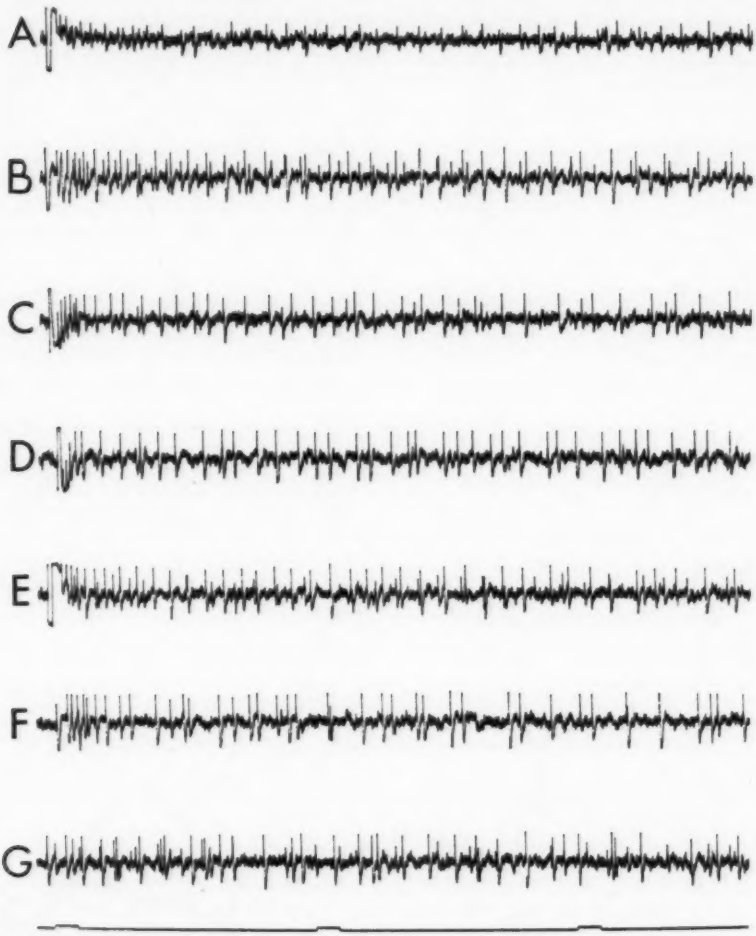

FIGURE 10. Receptor activity recorded through the side wall of one of the large marginal hairs of the labellum of a protein-deprived female *Phormia regina*. A, Response to water. B, Response to 1 *M* fructose. C, Response to brain-heart extract. D, Response to 1 *M* NaCl. E, Response to brain-heart extract plus fructose. Mixture is 0.5 *M* with respect to fructose. F, Response to a mixture of brain-heart extract and NaCl. Mixture is 0.5 *M* with respect to NaCl. G, Response to an equimolar (0.5) mixture of NaCl and fructose. Time, 0.2 second.

Internal factors affecting protein intake

A complicated and still only partially understood hormonal relation exists between the ovaries, the corpus allatum, the corpora cardiaca, and the median neurosecretory cells of the brain. Since preference tests revealed that the pattern of protein intake differs in the male and female and is correlated with ovarian changes, the ovaries and various endocrine organs were removed surgically and protein intake measured.

Removal of the ovaries did not alter the pattern of protein and carbohydrate intake (Fig. 8). Although the total volume of protein taken was less than that of the normals (but not of controls that had had a sham operation), it still showed the typical peak in the first six-day period and still exceeded that of males. There was a slight but not significant decrease in total carbohydrate intake (Table I).

When the corpus allatum was removed, eggs failed to develop; but there was no marked change in the pattern of protein and carbohydrate intake (Fig. 9). The reduction that did occur was matched by that of animals receiving sham operations (Table I).

Flies from which the median neurosecretory cells had been removed failed to survive longer than two weeks. During that period, however, the females exhibited the usual protein peak. Furthermore, in the ring tests they behaved like normal flies in their reactions to protein and carbohydrate.

Discussion

As Strangways-Dixon (1959, 1961) had already demonstrated with *Calliphora*, the volume of protein ingested by female blowflies is greater than that ingested by males and varies with phases in the reproductive cycle. It is clear from studies with *Phormia* that variations in ingestion reflect differences in feeding preferences. Thus, when a fly is given equal opportunity to ingest protein and carbohydrate, it refuses or ingests only a small amount of carbohydrate immediately after emergence and following each period of oviposition. At these times a large volume of protein is taken. The preference is accentuated if the fly is temporarily denied protein during these critical periods.

Observing the behavior of the females deprived of protein one gets the impression that there is a distinct "hunger" for protein. This reaction is particularly interesting because protein by itself is inadequate for survival. Thus, during these periods the fly eschews a nutritionally adequate diet (carbohydrate) for one which meets its reproductive requirements. From an evolutionary point of view, reproduction of the species takes precedence over survival of the individual.

The relationship between protein and carbohydrate ingestion is, however, not absolute. There is a strict dependence on the stimulating effect of the two substances on the sense organs. If, for example, the choice is between a highly stimulating carbohydrate (*e.g.*, 1.0 *M* sucrose) and protein, ingestion is biased in favor of the carbohydrate. Conversely, if the carbohydrate is only weakly stimulating (*e.g.*, 0.001 *M* sucrose), the ingestion of protein is tremendously augmented. Changing the kind of protein also alters the volumes taken. Homogenized liver is preferred to brain-heart infusion and both are preferred to hemoglobin; yet each suffices for egg production (although hemoglobin is the least satisfactory).

At first thought it would appear that the characteristic odors of these materials might be a deciding factor in choice and ingestion. Anosmic flies, however, are no different in their reactions toward protein and carbohydrate than normal flies. This finding is in agreement with data reported by Dethier and Chadwick (1947) and Evans and Barton Browne (1960). The fact that flies can detect the difference between protein and carbohydrate before ingesting them is inescapable. Observations of the behavior toward rings of solutions drawn on paper support the conclusion that discrimination is accomplished through the agency of the contact chemoreceptors on the legs and mouthparts.

Recent behavioral, electrophysiological, and histological work has shown that the innervation of the labellar hairs of *Phormia* is neither so simple nor uniform as originally believed (Dethier and Evans, 1961; Mellon and Evans, 1961; Larsen, personal communication). Some hairs have three neurons; others, four and possibly five. In addition to a neuron responding to bending there is one sensitive to water, one to sugar, and one to monovalent salts. While no specific protein receptor has been found thus far, there are differences among hairs with respect to the protein tested. One type of hair responds to carbohydrate and protein while another type responds only to carbohydrate. Protein appears to block activity in this hair. Both types respond to water and to sodium chloride. Although additional studies will be required before it is possible to assign protein sensitivity to a particular neuron, the point of importance now is that the fly has a peripheral mechanism for protein and carbohydrate discrimination.

Since it is true that the fly's response to protein and to carbohydrate varies concurrrently with events in the reproductive cycle, it must be inferred that the sensory contribution to behavior varies. The two most likely alternatives as to the level where changes occur are: the sense organs themselves; some intermediate level in the central nervous system. Either the relative sensory thresholds to protein and carbohydrate change periodically or some change occurs in the central nervous system where the sensory information is processed. There is as yet no direct information relating to sensory thresholds although by analogy with other sensory modalities in the fly it is unlikely that changes occur here. The alternative is that changes associated with protein metabolism alter integration by the central nervous system of sensory information coming to it. There are a number of ways in which changes occurring in the reproductive cycle might be linked with sensory input.

There are complicated and still only partially understood relations between the endocrine system, the reproductive system, and protein metabolism (*cf.* Wigglesworth, 1954; Strangways-Dixon, 1959). In *Phormia*, as in certain other blowflies, females fed carbohydrate alone are unable to bring the eggs to full development. If protein is provided, eggs develop fully, but they will not be laid unless copulation occurs. If the corpus allatum or medial neurosecretory cells are removed, egg development will progress only as far as in carbohydrate-fed flies.

It is conceivable, therefore, that differences in the fly's behavior toward protein and carbohydrate might be determined by one or more hormones or by changes in protein titer. The two possibilities for endocrine control are that the hormones increase sensitivity to protein or decrease it. If the first alternative is true, removal of the corpus allatum or medial neurosecretory cells should cause changes

in behavior toward protein. Neither changes in behavioral threshold nor difference in intake between operated and normal flies occurred. It might be argued that there was enough residual hormone to prevent a change; however, this appears unlikely since there was obviously not enough to permit egg development. Furthermore, allatectomized flies could be held for eight days on carbohydrate and still show a preference when presented with protein. These experiments rule out any hypothesis that hormones are *directly* concerned with nervous activity leading to protein ingestion.

In no case did removal of any of the endocine glands or of the ovaries prevent the fly from showing a protein peak shortly after emergence. In all cases subsequent protein peaks were absent. Removal of the ovaries alters protein levels simply because there are no developing eggs to create protein demands. Removal of the corpus allatum or medial neurosecretory cells alters protein levels because the absence of hormones also prevents egg development. Additionally, removal of the medial neurosecretory cells interferes with protein metabolism (Thomsen and Møller, 1959). Insofar as protein is concerned Strangways-Dixon (1959, 1961) obtained similar results with *Calliphora*. He did report, however, that allatectomy depressed carbohydrate intake, and this did not occur with *Phormia*.

The occurrence of an initial peak in protein ingestion unaffected by allatectomy, removal of the ovaries, or removal of the medial neurosecretory cells might be explained by assuming that all flies emerge from pupation with a protein deficit and that it affects behavioral threshold. This would explain the occurrence of initial protein ingestion in both sexes. In males the quantity is small and soon approaches zero. In the newly emerged female the initiation of egg development causes withdrawal of protein from the fat body (Strangways-Dixon, 1959) thus increasing the deficit. Accordingly, if females are denied protein for the first six days, the deficit becomes acute and sensitivity should increase (as indeed it does). In the males there is no such increase. Greenberg (1959) stated that there was no difference in the protein consumption of male and virgin female houseflies; however, his conclusion was based upon a comparison of mean daily intake, a measurement which tends to minimize the differences occurring shortly after emergence. After this time there are no pronounced differences. If a female is mated, a protein deficit develops, and the cycle is repeated.

The point at which protein deficit influences sensory input is at present unknown. It is not merely a matter of body pressure or osmotic relations in the body cavity because injection of carbohydrate, water, or hypotonic salt into the hemocoel fails to alter the pattern of protein ingestion. It is unlikely that it is any aspect of gut physiology because transection of the recurrent nerve, which innervates the gut, also fails to alter protein ingestion. When protein-deficient females are placed in a choice situation after recurrent nerve transection, they become hyperphagic, but do so by ingesting carbohydrate rather than protein.

The assistance of Mr. D. Mellon is gratefully acknowledged.

Conclusions

There is a difference in the pattern of protein feeding by the two sexes of the blowfly *Phormia regina* Meigen. Males, whether mated or not, gradually increase

their intake from the time of emergence until the fourth to eighth day. Thereafter little protein is taken. The pattern is similar for virgin females, but the volume ingested is greater. Mated females increase their protein intake after each batch of eggs is laid. If females are denied protein at times when the intake would normally be great, they show a decided preference for protein over carbohydrate in a choice situation. Choice is mediated by the contact chemoreceptors; odor is not a factor. The peak in protein ingestion that occurs after emergence is not altered by removal of the ovaries, corpus allatum, or medial neurosecretory cells. Subsequent peaks are abolished by any procedure that prevents egg development. Changes in feeding behavior are correlated with changes in protein levels which in turn are related to hormonal and reproductive cycles. It appears unlikely that hormones are directly concerned with nervous activity leading to protein ingestion.

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A HISTOPHYSIOLOGICAL STUDY OF THE RAT DIAPHRAGM

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Though some extensive studies have been made on the general morphology and physiology of the mammalian diaphragm as a whole, sufficient attention has not been paid to the regional and cellular organization of this organ. Morphologically, three distinct regions, namely vertebral (dorsal), costal (lateral) and sternal (ventral) have been described in man (Johnston and Whillis, 1949). But little information is available on the cellular components which constitute these regions, with regard to their histological features and metabolic activities. In a recent study Frunder (1954) observed that the oxygen uptake in the posterior and anterior parts of the diaphragm differed with regard to their glycogen content, and pointed out possible errors in respiration studies with selected parts of the diaphragm.

Recent studies conducted in our laboratories on the structure and physiology of mixed muscles, such as the pigeon breast muscle, which have been reviewed by Drummond and Black (1960), have shown the existence of two types of fibers: one broad, white and glycogen-loaded, adapted for an anaerobic metabolism in which glycogen is the chief fuel for energy; and a narrow, red fat-loaded variety for aerobic metabolism in which fat forms the chief fuel. The diaphragm also being a mixed type of muscle, it was thought desirable to carry out similar studies on its different regions with a view to correlating structure with function. In our present investigation the three regions of the rat diaphragm have been studied with regard to the nature, number and distribution pattern, diameter, metabolite load and concentrations of enzymes (lipase and succinic dehydrogenase) of the constituent fibers.

Materials and Methods

Freshly collected wild rats (*Rattus norvegicus*) were used as the material in the present investigation. The rats were decapitated and the whole diaphragm was quickly removed and spread on a clean filter paper. The three regions were demarcated and cut out separately. For histochemical observations thin strips of the tissue were then cut in line with the orientation of the fibers. For the quantitative estimations, each separate region was pooled from a number of individuals.

Fiber count and fiber diameter

Thin, frozen, hand sections of all the three regions, were separately cut out, spread on clean, dry slides and mounted in glycerine jelly. The respective numbers of red and white fibers within a certain lens field were counted. With the aid of an ocular eyepiece and micrometer scale the diameter of the fibers was determined.

Fat: histochemical localization and quantitative estimation

The tissue was fixed in Baker's calcium formol (Baker, 1946) for 24 hours and washed in running tap water for the same time and embedded in 20% gelatin. Thirty- to forty-micron sections were cut on a freezing microtome and stained for fat, using the Sudan Black B stain.

As already mentioned the material from a single individual was found to be insufficient for quantitative estimations and so the material from a number of individuals, irrespective of sex, was pooled together. It was then dehydrated by drying in a hot air oven at 100° C. The total fat content was estimated by the Soxhlet extraction method, using a 1 : 1 alcohol-ether mixture.

Glycogen: histochemical localization and quantitative estimation

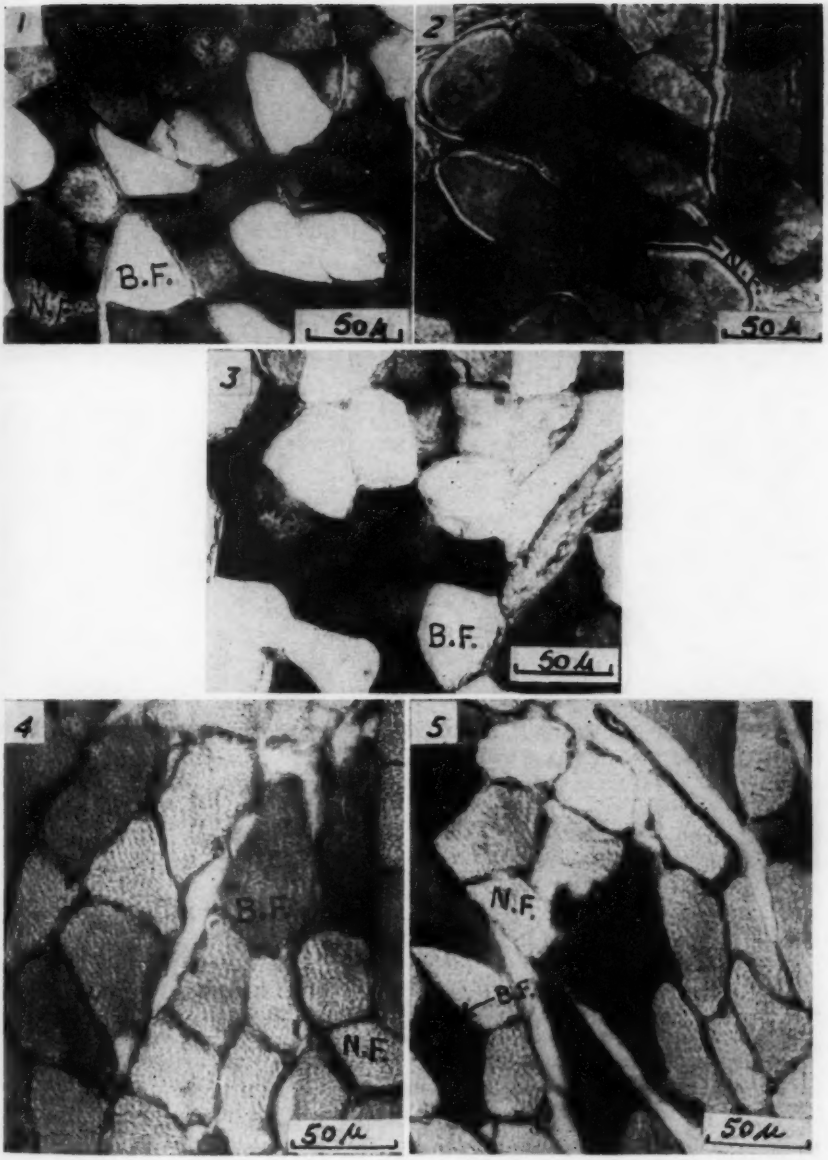
The tissue was fixed in cold alcoholic picroformol for 24 hours and embedded in paraffin wax of melting point 56 to 58° C. Sections 10 to 12 μ thick were cut and stained for glycogen by the periodic acid Schiff's reagent (Pearse, 1960).

Glycogen was estimated quantitatively by the micro method of Kemp *et al.* The material was pooled and treated with 80% methanol for the removal of glucose. The insoluble part was deproteinized and the clear supernatant hydrolyzed by concentrated sulphuric acid. The intensity of the pink color was measured on a Klett-Summerson photoelectric colorimeter, using a 520 mμ filter. The amount of glycogen present in the material was directly read from the standard graph. The results are expressed as μg. glycogen/100 mg. wet tissue.

Lipase: histochemical localization and quantitative estimation

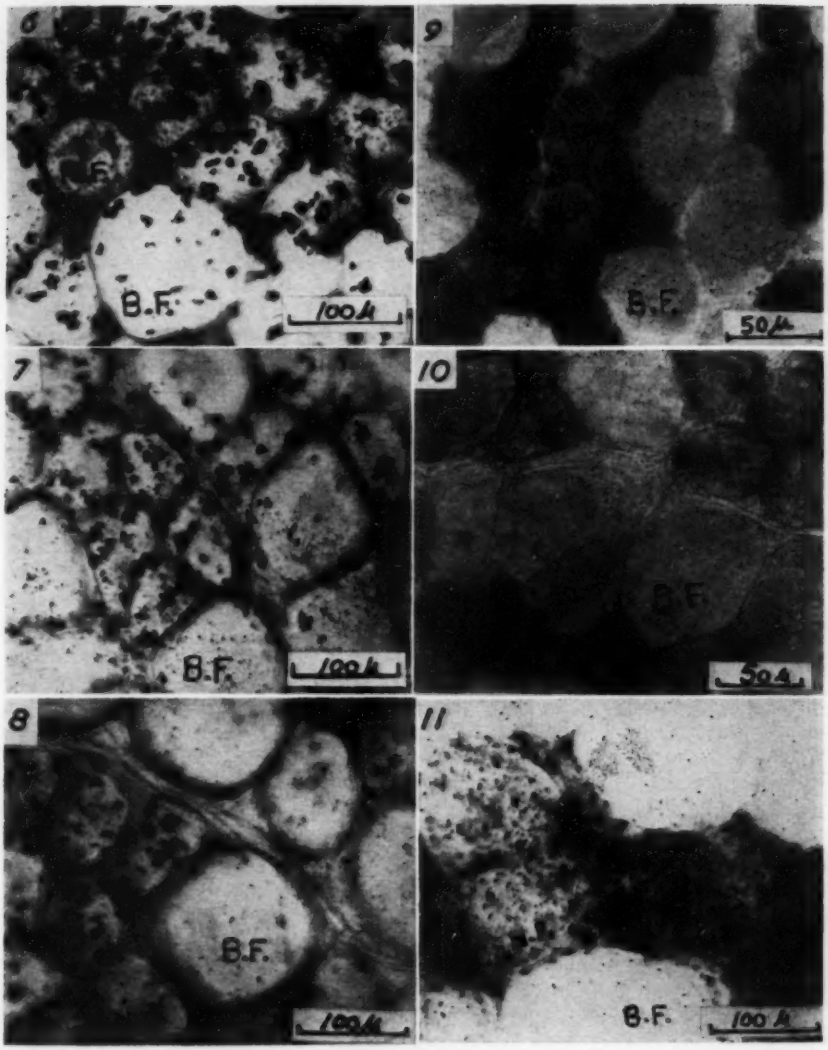
The lipase activity in the two types of fibers was studied histochemically using an improved technique (George and Iype, 1960). Thin frozen sections were cut according to the method of George and Scaria (1958) and dried on clean albumenized slides at room temperature. The sections were fixed in cold 6% neutral formalin for 3 to 4 hours, washed well in running tap water for about an hour and rinsed well with distilled water. A thin coating of gelatin (5%) was then applied on the sections and fixed in 6% neutral formalin for half an hour. It was washed in tap water for another half an hour. The sample sections were incubated for 12 to 16 hours in the incubation medium with "Tween 80" as substrate. The control sections were boiled for 10 minutes, coated with gelatin and incubated along with the sample sections. After incubation, the sections were washed well with distilled water and then with warm water (40° C.), so that the gelatin coating was completely removed. The sections were then treated with lead nitrate for half an hour and then with dilute yellow ammonium sulphide. According to the modifications made by George and Iype (1960), a final step of rinsing the sections in 1–2% acetic acid was also adopted for clearing the sections. The brownish black precipitates of lead sulphide formed as the result of the enzymic activity were seen very prominently.

The quantitative estimation of lipase activity was carried out according to the method of Martin and Peers (1953) using a Warburg manometric apparatus. The results obtained are presented as μl. CO_2 produced per mg. protein per hour.



FIGURES 1, 2, and 3. Transverse sections of the dorsal, ventral, and lateral regions, respectively, of the diaphragm stained with Sudan Black B for fat. The broad fibers (B.F.) are less sudanophilic than the narrow ones (N.F.).

FIGURES 4 and 5. Transverse sections of the dorsal and lateral regions, respectively, of the diaphragm stained with PAS for glycogen. The broad fibers contain more glycogen than the narrow ones.



FIGURES 6, 7 and 8. Histochemical localization of lipase activity in the fibers of the dorsal, ventral and lateral regions, respectively. The enzymic activity in the narrow fibers is more than in the broad ones.

FIGURES 9, 10 and 11. Histochemical demonstration of succinic dehydrogenase activity in the fibers of the dorsal, ventral and lateral regions. The deposition of the diformazan granules is more in the narrow fibers.

The protein content of the tissue was estimated by the micro-Kjeldahl steam distillation method (Hawk *et al.*, 1954).

Succinic dehydrogenase: histochemical localization and quantitative estimation

Thin frozen sections of the three regions of the diaphragm were separately cut out and the histochemical demonstration of succinic dehydrogenase was carried out by an improved method of George and Talesara (1961) using neotetrazolium chloride as the hydrogen acceptor. The sections were transferred to cold 0.1 *M* phosphate buffer (pH 7.4) for 10 minutes to destroy the endogenous substrates. They were then transferred to the incubation medium consisting of 0.1 *M* phosphate buffer (pH 7.4) 2.5 ml., 0.5 *M* sodium succinate 0.6 ml., 0.004 *M* aluminium chloride 0.5 ml., neotetrazolium chloride (3 mg. per ml.) 1 ml., methylene blue (1 mg. per ml.) 0.1 ml., 0.6 *M* sodium bicarbonate 0.3 ml. and 0.005 *M* magnesium sulphate one drop. The incubation was carried out under strictly anaerobic conditions by passing a mixture of nitrogen (95%) and carbon dioxide (5%) through the incubation medium at 37° C. for 5 to 10 minutes. The purple granules of the diformazan formed indicated the sites of the enzymic activity.

The enzyme activity was assessed quantitatively by the colorimetric method of Kun and Abood (1949). The results are expressed as μg. formazan formed per mg. dry weight per two hours.

Results

Histochemical

Fat. In all the three regions it was found that the broad fibers were less sudanophilic than the narrow ones (Figs. 1, 2, 3).

Glycogen. In the dorsal and lateral regions, the broad white fibers were found to contain more glycogen than the narrow ones (Figs. 4, 5). In the ventral region, however, the glycogen content was so low that satisfactory histochemical demonstration was not possible.

Lipase. The level of lipase activity in the three regions differed according to the nature and distribution of the fibers present, the broader fibers having less lipase activity (Figs. 6, 7, 8).

Table I

Giving the relative distribution, metabolite load and enzyme (lipase and succinic dehydrogenase) activity in the two types of fibers in the three regions of the rat diaphragm

Region of the diaphragm	Number of fibers in % per unit area		Ratio of the fibers B:N	Diameter of the fibers in μ		Diameter ratio B:N	Area covered per unit area in μ^2	
	Broad	Narrow		Broad	Narrow		Broad	Narrow
Dorsal (vertebral)	29.23	70.76	1:2.4	78.30	40.50	1:1.9	32,230	21,530
Lateral (costal)	32.29	67.70	1:2.1	90.72	43.20	1:2.1	40,100	19,060
Ventral (sternal)	32.24	67.78	1:2.1	49.40	37.80	1:1.3	18,890	23,020

TABLE I—(*Continued*)

Region of the diaphragm	Area ratio B:N	Glycogen μg./100 mg. wet tissue	Fat % dry weight	Lipase μl. CO_2/mg. protein/hour	SDH μg. formazan/mg. dry weight/2 hours
		S.D.	S.D.	S.D.	S.D.
Dorsal (vertebral)	1:0.7	232.72 ± 24.95	16.19 ± 1.43	41.54 ± 5.52	10.97 ± 2.45
Lateral (costal)	1:0.5	297.42 ± 21.37	13.17 ± 1.55	24.55 ± 4.25	14.16 ± 3.22
Ventral (sternal)	1:1.2	127.42 ± 15.68	21.41 ± 0.80	29.79 ± 3.11	8.06 ± 2.81

Succinic dehydrogenase. The broad fibers showed considerably less enzyme activity than the narrow ones, in all the regions. In the lateral region, however, the narrow fibers, even though broader than the narrow fibers of the other two regions, showed high enzyme activity (Figs. 9, 10, 11).

Quantitative

The data obtained from the quantitative estimations are given in Table I. From Table I it is clear that each of the three regions has its own characteristics. The dorsal region has the highest number of narrow fibers and the least number of broad fibers, the diameter of the narrow fibers being less than that of the narrow fibers of the lateral region but higher than those of the ventral region. The diameter of the broad fibers is, however, less than that of the broad fibers of the lateral region but more than that of the broad fibers of the ventral. The area covered in a unit space by the broad fibers is less than that covered by the broad ones of the lateral and the area covered by the narrow fibers in the same space is less than that covered by the narrow ones of the ventral. The glycogen content is less than that in the lateral but more than that in the ventral. The fat content is higher than that in the lateral while it is less than that in the ventral. The dorsal region, again, has the highest lipase activity among all the three regions and succinic dehydrogenase activity less than that in the lateral but more than that in the ventral.

Of the three regions the ventral has the narrowest narrow fibers; narrowest broad fibers; the largest area covered by narrow fibers; has the least glycogen content; highest fat content; high lipase activity, much higher than the lateral but lower than in the dorsal, and the least succinic dehydrogenase activity.

The lateral region, on the other hand, possesses the broadest broad fibers; broadest narrow fibers; largest area covered by the broad fibers; highest glycogen load; least fat load; least lipase activity but highest succinic dehydrogenase activity.

DISCUSSION

That the diaphragm is a mixed type of muscle, having fibers of different sizes, is well known. Gunther (1953) studied the human, dog, and rat diaphragms and distinguished two types of muscle fibers, tetanic and tonic ones, the latter amounting to 10% in the human, 42% in the rat and 5% in the dog. He thus suggested a functional difference in the two types of fibers. Recently Nachmias and Padykula

(1958) in their histochemical study observed in the rat diaphragm two types of fibers, those with smaller average diameter having greater succinic dehydrogenase activity and others with larger diameter with lesser enzymic activity. In the cat diaphragm, however, they described three types of fibers, the third type being characterized by intense uptake of Sudan Black B in the subsarcolemmal position.

In the present study, two distinct types of fibers, namely a broad white and a narrow red, similar to the two types described in the pigeon breast muscle (George and Naik, 1957), existing side by side in all the three regions of the diaphragm, have been recognized. However, the distribution pattern of these two types of fibers, in contrast to that of the pigeon breast muscle (George and Naik, 1959), does not show any definite design and appears to be at random. It was also observed that the diameter of the fibers, unlike that of the pigeon breast muscle, showed a high degree of variation in each of the two types. Nevertheless, from our histochemical observations it is seen that there is a correlation between the diameter of the muscle fiber and its color, metabolite load, mitochondrial content and enzyme concentrations. Such a relationship has been shown in the pectoralis muscle of birds (George and Naik, 1957; George and Scaria, 1958a; George and Talesara, 1960, 1961), bats (George, Susheela and Scaria, 1958) and the flight muscles of insects (George and Bhakthan, 1960a, 1960b). However, in the case of the fibers of the diaphragm in which the narrowest white fiber and broadest red fiber have diameters of 49 μ and 43 μ, respectively, the above correlation was not so marked as in the case of the broad and narrow fibers of the pigeon breast muscle. In the bat pectoralis such variations in diameter were observed (George, Susheela and Scaria, 1958). The fibers of the pigeon breast muscle are therefore to be regarded as an instance of extreme specialization achieved in the differentiation of the two types of fibers.

In the diaphragm the broadest white fibers are distinctly loaded with glycogen and contain less mitochondria (as evinced by the staining for lipid) and less lipase and succinic dehydrogenase activity. In the case of the narrower white fibers and broader red fibers, however, it was not possible to arrive at definite conclusions based on histochemical observations. It also became clear that instead of the high degree of specialization resulting in the two distinct fiber types as in the pigeon breast muscle, in the diaphragm, the differentiation of the component fibers achieved a greater extent of variability in structure and perhaps function too. On the other hand, the diaphragm achieved a specialization in having three distinct regions, which is revealed from our histochemical and quantitative observations.

The fact that the dorsal region possesses the highest number of narrow fibers, high fat content, highest lipase activity and high succinic dehydrogenase activity suggests a predominance of fat metabolism involving the use of fat as fuel for muscular contraction. And it should be mentioned here that the utilization of fat by the diaphragm has been demonstrated by Wertheimer and Ben-Tor (1952). It has also been observed (George and Susheela, 1961) that during starvation there is a six-times actual reduction of fat in the dorsal as well as in the ventral regions over the lateral region, of the rat diaphragm.

The predominance of fat metabolism also appears evident in the ventral region where there is the highest fat content and high lipase activity. But this region comparatively has the lowest level of oxidative capacity in terms of succinic de-

hydrogenase activity. This indicates that in this region there is more of building up of fat from fatty acids for storage than fat metabolized for energy. Some indirect evidence for the possibility of fat being built up as energy store at sites rich in lipase activity, but poor in oxidative enzyme activity, has been recently presented by George and Iype (1960). They have demonstrated in the sheep heart high lipase activity and very low succinic dehydrogenase activity in the Bundle of His, and suggested the possibility of this site in the heart being a center for lipogenesis. They also demonstrated high concentrations of both the enzymes in the myocardium which is known to utilize fat as the major fuel for the activity of the heart. Further, the works of Cogan and Kuwabara (1957) and Kuwabara and Cogan (1960) have shown that the Purkinje fibers, the structural units of the Bundle of His, have the capacity to synthesize sudanophilic fat.

From the characteristics of the lateral region mentioned earlier, it is obvious that this region is specialized for carbohydrate metabolism in which glycogen forms the fuel for muscular contraction. The very high succinic dehydrogenase activity, even higher than what is seen in the fat-loaded dorsal and ventral regions, is certainly indicative of high oxidative metabolism involving the rapid oxidation through the Krebs cycle of the products of the Emden-Meyerhof cycle. Another possibility is that, under conditions of prolonged rapid respiratory activity, this region might well be capable of oxidizing fatty acids transported from the other regions of the diaphragm and/or from other sites in the body where fat is stored. In this context it may be mentioned that a recent study of the blood supply of the rat diaphragm (Beck and Baxter, 1960) has revealed the existence of an elaborate and copious system of circulation in this organ. Of the three regions, the lateral region has the major blood supply while the ventral has the least. Moreover, the blood supplies to the lateral and dorsal regions are more directly from the aorta through the phrenic arteries, than the ventral which receives blood from a small branch of the internal mammary artery. Their study has also shown the remarkable consistency in the main features of the venous drainage and the presence of an anastomatic system of veins in the diaphragm, connecting the inferior vena cava and azygous veins.

The present investigation on the diaphragm has revealed more of the complex nature of this organ than it has actually contributed to the elucidation of our knowledge of its structure and physiology. To that extent we feel amply rewarded. However, more extensive histophysiological and biochemical studies on the different regions are called for and are in progress.

One of us (A.K.S.) is indebted to the Council of Scientific and Industrial Research (Govt. of India), for the award of a Junior Research Fellowship.

Summary

1. The nature and distribution of two types of fibers in the rat diaphragm, with respect to its dorsal (vertebral), lateral (costal), and the ventral (sternal) regions, have been studied.

2. Histochemical as well as quantitative studies on the fat and glycogen contents and the enzymic activity (lipase and succinic dehydrogenase) have been made in the above three regions.

3. It is concluded that the narrow red type of fibers is well adapted for aerobic metabolism, involving mainly the oxidation of fat for energy, and the other, the broad white variety, for anaerobic metabolism where glycogen is the chief fuel. The significance of the regional differences in the two types of fibers in the physiology of the diaphragm is discussed.

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THE EFFECT OF THIOUREA, ADMINISTERED BY IMMERSION OF THE MATERNAL ORGANISM, ON THE EMBRYOS OF LEBISTES RETICULATUS, WITH NOTES ON THE ADULT GONADAL CHANGES[1]

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The terms oviparous, viviparous and ovoviviparous are commonly found in the literature discussing embryology and physiology. Oviparous and viviparous may be defined with relative ease and the interpretations of most authorities are in agreement. Briefly, oviparous refers to animals that deposit eggs outside of the body, while viviparous applies to animals that retain the ova and have their development within the soma. The latter term is applied to those forms having highly specialized structures, such as placentae, which attach the developing young to some part of the reproductive system of the adult in order to provide an avenue of material exchange, including nutritive materials for the growth of the embryo during the greater part of its development within the parent. The third term, ovoviviparous, has been variously defined and is less definite. It is agreed that in those forms described as being ovoviviparous the young are retained within the maternal reproductive system and leave the mother's body fully formed, viable and without any surrounding membranes. There does not appear to have been a permanent attachment, however, and the degree of dependence of the young on the parent is a moot question. The eggs of many species referred to as ovoviviparous are macrolecithal, and many authors believe the produced egg contains enough yolk to meet the nutritive needs of the developing young until it leaves the parent. The young is materially independent of the adult and uses the latter only as a harbor while developing. The distinction between viviparity and ovoviviparity is therefore classically based on anatomical and physiological differences.

A true anatomical uterus and placenta, which permits a firm attachment of the embryo to the parent, is wanting in teleosts. Structures and modifications of parts of the reproductive tracts (of both male and female) which permit retention and internal hatching of the young have been reported to occur in diverse orders of the class (Turner, 1942, 1947). In some of the species studied it has been shown that both parent and young may possess structures whose histological and cytological make-up, spatial arrangement and time of appearance suggest a material exchange between parent and young (Blake, 1867, 1868; Eigenmann, 1892; Fraser and Renton, 1940; Lane, 1903; Mendoza, 1937, 1938, 1939, 1940, 1941, 1943; Purser, 1938; Turner, 1933, 1936, 1937a, 1938a, 1938b, 1940a, 1940b, 1940c,

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1940d; Wyman, 1854). Some physiological studies (Bailey, 1933; Scrimshaw, 1944, 1945) comparing egg and embryo weights indicate the supply of materials to the young by the parent in certain species of teleost.

This paper reports the results of a study designed to reinvestigate the question of exchange between parent and young in one teleost, the guppy, *Lebistes reticulatus* (Peters), often referred to as ovoviviparous, by using the anti-thyroid chemical, thiourea, which is known to have the ability to cause the alteration of the morphology and physiology of the thyroid gland. It was thought that the chemical, when supplied to the adult, would produce discernible changes in the young if a material transfer does exist. *Lebistes reticulatus* was used as the experimental animal because of its hardiness, macrolecithal egg, and the presence of structures (Purser, 1938) that could possibly aid in the transfer of substances.

The author expresses his appreciation to Dr. Harry A. Charipper for the kind advice and guidance given during the course of this project. Thanks are extended to Dr. Charles M. Breder, Jr. and to Dr. Ross F. Nigrelli for their valuable suggestions. The major part of this work was performed in the Laboratories of the Department of Fishes and Aquatic Biology of the American Museum of Natural History, New York.

Materials and Methods

Seventy-five brood-bearing *Lebistes reticulatus* were utilized in this work. They were selected from a larger group of females exposed continuously to males soon after birth up to and one day after the delivery of their third brood. The fish were all of approximately equal size and found to present young at 28–31-day intervals. They were divided into 5 groups of 15 each as follows:

Group C: Received no treatment, served as controls.

Group 1D: Placed in a 0.04% thiourea solution one day after the delivery of their third brood. This timing exposed the adults to the solution at the time a group of ova were maturing, during fertilization and the entire ontogeny of the young.

Group 1W: Placed in a 0.04% thiourea solution one week after the delivery of their third brood. This timing exposed the adults to the solution at approximately the time of fertilization and during the entire ontogeny of the young.

Group 2W: Placed in a 0.04% thiourea solution two weeks after the delivery of their third brood. This timing exposed the adults to the solution during the latter two-thirds of the ontogeny of the young.

Group 3W: Placed in a 0.04% thiourea solution three weeks after the delivery of their third brood. This timing exposed the adults to the solution during the last one-third of the ontogeny of the young.

The young of the groups were labeled with a "Y" after the parents' group, giving the symbols CY, 1DY, 1WY, 2WY, 3WY.

Homotypic conditioned water was used in the preparation of the thiourea solutions and in the tanks containing control fish. Each animal was kept separate in a one-gallon container without plants, "scavengers" or aeration. Solutions were

changed semi-weekly. All fish, except as noted below, were allowed to present their broods. To insure against a long exposure of the newborn young to the thiourea solution all tanks were checked four times a day and any young found were fixed immediately. In only two cases, were broods presented during the night. These young may have been in the solution for a maximum of 10 hours. Seventeen non-control fish were observed to be delivering young and were sacrificed during the birth process. All other adults were killed when young were found in the tanks. Water temperatures ranged from 72 to 78° F. throughout the experiment. A portion of the controls and of each of the treated groups coexisted at all times. This arrangement served as a check on possible seasonal effects. Tanks containing control fish were always adjacent to the tanks containing treated fish in order to assure an equal distribution of light to all. Daylight was supplemented with artificial light during the late fall and winter months.

The fish were killed and fixed in Bouin's solution. As soon as movement ceased the adults were removed from the liquid, their abdominal wall was slit and they were reimmersed in the agent. This procedure allowed a more rapid and direct penetration of the fixing fluid into the body cavity. Adult fish were decalcified with nitric acid plus phloroglucin; young were not decalcified. Xylol was used as the clearing agent in preparation for embedding in 56–58° C. Histowax. Sagittal serial sections of the entire fish were cut at five micra and stained either with Harris' hematoxylin and eosin or a modified Masson procedure.

Thyroid cell height was measured with a calibrated ocular micrometer. A minimum of 100 cells was observed for each fish, involving as many different follicles as possible. The data obtained were subjected to statistical analysis and their significance determined.

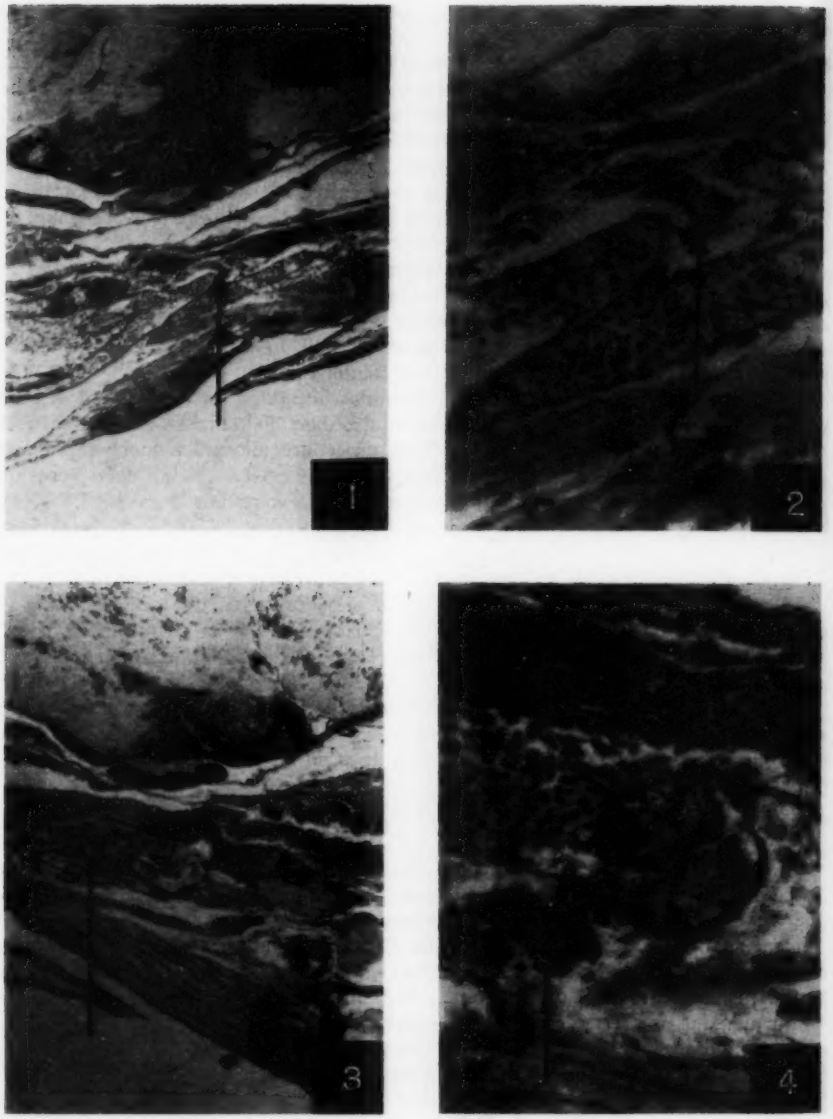
Brood sizes and intervals of all individuals of all groups were recorded. The ovaries of all adult fish were examined macroscopically and microscopically and the contained ova were grouped according to size.

Results

I. *Thyroid*

A. *Normal.* The thyroid gland of the normal *Lebistes reticulatus* was found to be a diffuse and unencapsulated organ consisting of many separate follicles in the connective tissue of the floor of the mouth. They are arranged alongside the ventral aorta and other blood vessels and extend from the anterior part of the mandible to the gill region; see Figures 1 and 2. Thyroid epithelia were not found in any other part of the body. Without exception the follicular cells are of a squamous or a very low cuboidal nature, as seen in Figure 5. The contained colloid is dense, homogeneous and fills the interior of all the follicles. The picture is essentially the same for the adult and the newborn.

B. *Treated Adults.* After immersion in the thiourea solution, hyperplastic changes were noted in all fish. Follicles increased greatly in size with very great variation. Without exception, follicles increased in number and extent. Some were found posteriorly to the level of the heart, throughout the connective tissue of the entire lower jaw, crowded around the blood vessels, and in the gill region. Members of group 1D showed the greatest degree of metastasis. In this group, thyroid cells were found in the myocardium in 12 cases, in the kidneys in 9 cases, in



Tissues represented by Figures 1–7 were stained with Harris' hematoxylin and eosin, Figure 8 represents an unfixed, unstained young.

Plate I

FIGURES 1 and 2. Section of the floor of the mouth of a normal young showing few thyroid follicles. Figure 1, × 132; Figure 2, × 525.

FIGURES 3 and 4. Section of the floor of the mouth of a young from a thiourea-treated fish showing massing of cells and afollicular arrangement of cells near the blood vessels. Figure 3, × 132; Figure 4, × 525.

Plate II

FIGURE 5. Thyroid follicle from an untreated fish. × 525.
FIGURE 6. Thyroid follicle from a thiourea-treated fish. × 525.
FIGURE 7. Heart of a thiourea-treated fish showing follicles in the myocardium. × 132.
FIGURE 8. Aborted young from a thiourea-treated fish. × 15.

the intestines and spleen in 8 cases. Scattering of thyroid tissue took place to a lesser extent in fish of groups 1W and 2W. Group 3W did not show any atypical location of thyroid cells. The invading tissue in the new areas was arranged in small follicles or was massed in layers approaching an afollicular disposition. Colloid was reduced in amount. It was of an acidophilic nature and appeared foamy and vacuolated.

Thyroid cell height was markedly increased, the amount increasing with the time of exposure (Fig. 6). In all cases, the difference between the experimental and control animal data proved to be statistically significant (see Table I). Cytoplasmic staining was more intense. The nuclei increased in size, became more vesicular and basal in position. Nucleoli became more prominent.

TABLE I

Experimental condition of the fish and thyroid cell height

State of immersion	Mean thyroid cell heights (μ)	S.E.*
Control—adults	2.71	0.010
Young male	2.22	0.008
Young female	2.33	0.009
1 day—adults	7.21	0.031
Young male	9.66	0.021
Young female	9.81	0.027
1 week—adults	6.70	0.032
Young male	9.55	0.022
Young female	9.69	0.021
2 weeks—adults	6.52	0.019
Young male	9.04	0.017
Young female	9.22	0.019
3 weeks—adults	6.17	0.014
Young male	8.60	0.020
Young female	8.84	0.015

* Standard error (standard deviation of the mean): S.E. $= \sqrt{\frac{Ed^2}{n(n-1)}}$.

C. *Young from treated adults.* All the young from all groups showed extensive invasion of new areas by thyroid cells. The gill regions, heart, kidneys, spleen and intestines contained thyroid tissue. Myocardium invasion is seen in Figure 7. No one specimen showed metastasis to all sites, however. Follicular arrangement became the exception rather than the rule. Cells were massed in large groups without follicular arrangment, as seen in Figures 3 and 4. Colloid was scant or, in many cases, absent. Thyroid cell height was increased and in all cases proved to be significantly different from the controls.

II *Gonads*

A. *Normal adult.* It can be seen macroscopically as well as with a dissecting microscope that after the presentation of a brood the ovary contains a large number

of ova. Study and measurements showed that they could be arranged in groups. The oldest and most mature group consists of eggs measuring 0.8 to 1.5 mm. These are orange in color and rather transparent. In numbers they approximate the size of the last brood or may be slightly more numerous. A second group consists of ova measuring 0.3 to 0.5 mm. These immature eggs, in addition to being smaller, are white, dense and opaque. Their number is considerably larger than either the first group or the brood last presented. Eggs in a still more immature stage of development are also present. They are by far the most numerous. Schematic grouping of the latter is not feasible because of too great a variation in size, almost continuous gradation existing within this group. Definite intervals existed between the three major groups, however.

B. *Normal young.* Sexual distinction of newborn *Lebistes* cannot be accomplished macroscopically. The anal fin modification (the gonopodium) and the typical coloration is not obtained by the male for nearly two months. Histological study of the immature gonad does reveal the sex of the individual, however, and may be used to sex specimens.

TABLE II

Experimental condition of the fish and brood size, brood interval and ova development

	Brood size		Brood interval in days		0.8–1.5 mm. ova		0.3–0.5 mm. ova	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
Control	14.6	0.25	29.5	0.08	15.3	0.23	20.3	0.33
1 day	8.5	0.25	35.0	0.23	4.6	0.12	1.8	0.07
1 week	9.5	0.23	33.0	0.07	6.1	0.18	2.2	0.08
2 weeks	10.6	0.20	32.0	0.06	6.8	0.13	3.2	0.08
3 weeks	14.2	0.26	30.8	0.07	12.6	0.25	12.5	0.26

C. *Treated adult.* There is a very apparent decrease in gonadal activity. Without exception, all members of groups 1D, 1W and 2W presented a brood smaller in number than either of their previous broods or the average of their three previous broods. This is most evident in group 1D. Members of group 3W were not so affected, as shown in Table II. Three of the 15 members of group 1D and one member of the 15 in group 1W presented their young earlier than the expected date, that is, the date calculated from their previous performance and the average for the group. The aborted young were not viable. Their bodies were blackened and were undergoing resorption (see Figure 8). A statistically significant increase in the interval between the presentation of broods in a normal manner took place after exposure to thiourea (see Table II). The ovaries of members of groups 1D, 1W and 2W contained fewer than the anticipated number of large, orange, transparent ova. Their number was smaller than the number of young in the broods presented by the fish since the start of treatment with the anti-thyroid drug and smaller than either of its pre-thiourea broods. In addition, the number of ova of the 0.3-0.5 mm. group was markedly reduced. Ovaries of all controls contained more small ova than either the number of the young born in a brood or the eggs composing the more mature group. These data are included in Table II.

D. *Young from treated adults.* The gonads of all young from treated adults were developed sufficiently to permit sexing of the individual by means of histological examination.

Discussion

It has been known for a long time that some teleost fishes retain their young within their body. This live-bearing habit is found in widely distributed families. According to Turner (1947), there seems to be no relation to ecological conditions, geographic distribution or taxonomical relationship. Live-bearing species are found in cold or warm water, lagoons, swift running streams, lakes, caves, at muddy bottoms, at water surfaces and among rocks in fresh or salt waters throughout the world. Although the group of teleosts is old and has had time for evolutionary change, none of the species has evolved a true placental attachment, erosion of maternal tissue by the embryo or an anatomical uterus to harbor the young. The length of time of retention of the young is variable. Some species (for example, the scorpaenids) present their broods in a very immature state. Turner (1942) referred to this condition as "incipient viviparity." Other species present their young in a more developed state. Males of *Cymatogaster aggregatus* are sexually mature when born. The number of broods developing at any one time depends upon the species in question. Superfetation is found in a number of groups. In the extreme case of *Heterandria formosa*, as many as nine broods in different stages of development may be contained within the ovary. All of the live-bearers, of course, show internal fertilization. The sperm traverse the gonaduct to reach the ovary. Egg activation and fertilization may take place immediately or the sperm may be immobilized and stored for a period of months in the ovary and used to fertilize several succeeding broods, according to van Oordt (1928) and Purser (1938). In the latter case, part of the ovarian epithelium acts as a nurse cell. No rule can be stated to describe the site of fertilization, time of ovulation, or area utilized to contain the developing young. If fertilization is intrafollicular, the development may proceed either within the follicle, or the contents of the follicle may be evacuated into the ovarian cavity, or the zygote may be extruded immediately. Turner (1947) referred to the first type as "follicular gestation," the second as "ovarian cavity gestation." Both of these necessarily mean that ovulation follows the fusion of the egg and sperm. Superfetation is found to be superimposed upon follicular gestation; in this case all follicles are not emptied at the same time. Hatching may take place in the follicle, ovarian cavity or after expulsion from the adult's body.

There is no correlation between site of harboring and the amount of yolk contained in the egg. The deutoplasm may be abundant or relatively scarce in either follicular gestation or in ovarian cavity gestation. Neither has the amount of yolk been found to be correlated with gestation time. The yolk may be relatively abundant during a short gestation period or it may be at a minimum while the gestation period is lengthy. The latter finding, coupled with the existence of temporary structures (both maternal and embryonic) present only during the gravid condition, as well as changes in permanently established organs, suggests *a priori* a material exchange between adult and embryo may proceed.

Structural developments and organ changes of some kind and degree have been found to exist in all live-bearing fish so far studied, regardless of egg yolk contents.

In forms showing a macrolecithal condition and displaying follicular gestation, maternal changes are mainly a thinning of the follicle wall and greatly increased vascularity of that area. The embryonic vascular pericardial sac usually becomes enlarged and drawn over the head region to form a hood-like covering which definitely increases the embryonic vascular surface (Purser, 1938; Turner, 1940c). On the other hand, species whose embryos are equipped with a small yolk supply show a greater number and degree of modifications in both parent and young. The follicular lining, in addition to becoming highly vascular, develops a large number of vascular extensions very much like villi. These may make contact with and be closely applied to the surface of the embryo or be suspended in the fluid contained in the follicle. The pericardial sac is more highly expanded than in the previous case.

Species utilizing the ovarian cavity as the embryo-harboring site display a number of more complex structures. Following fertilization and while still within the follicle, the embryos possess for a short period of time the distended pericardial sac described for the follicular gestation embryos. When in the ovarian cavity the structure is lost and is replaced by extensions of the anal area called trophotaeniae (Mendoza, 1937; Turner 1933, 1937a). In the developing embiotocid fishes, the soft tissues between the ray ends of the vertical fins become vascular and extended (Blake, 1867, 1868). At this time the adult embiotocid cavity wall develops a number of vascular flattened processes which may become applied to the surface of the embryo gill or mouth area. The epithelial lining has been described as high columnar and secretory at this time (Mendoza, 1939, 1940). The spatial arrangement of these structures, the time of their appearance, and their structure certainly support the hypothesis of material exchange in the possessor. Turner (1940) has employed the term "pseudo-placentae" to describe them. The fluid within the cavity which bathes the embryos is said to contain degenerating cells and non-surviving embryos. Both the fluid and these cells may serve as a source of food materials (Turner, 1937a, 1947).

Scrimshaw attempted to provide more definite proof for an interchange of substances. He determined the dry and wet weights of embryos at various stages of development. In 1944, he found that the dry weight of the *Heterandria formosa* embryo increases from 0.017 mg. at the time of fertilization to 6.8 mg. at the time of birth. Wet weight measurements were not sufficiently accurate, for no way was found accurately to eliminate the excess water. Scrimshaw in 1945 applied this method of attack to many genera of Poeciliidae. He found that the dry weight of the intact follicle, determined at various stages of development, showed no significant weight change in 18 species. This is in marked contrast to the situation in oviparous fish where approximately one-third of the initial weight of the egg is used for maintenance during development. Accordingly, Scrimshaw concluded that in the species studied, the adult contributes positively to the embryo and that they receive as much nourishment quantitatively as they require for maintaining their metabolic rate.

In the present study, *Lebistes reticulatus,* a live-bearing Poeciliid was utilized. This species displays follicular gestation and the embryos are supplied with much yolk. *Lebistes* is often referred to as ovoviviparous and it is thought by some workers that the material exchange is nil or at most is restricted to gases. Purser (1938) studied *Lebistes* and found an extremely vascular area covering the yolk sac

and extending between the eyes and opercula and making its way over the head. This strap of tissue is almost completely covered with a vascular network. Purser referred to this development as a "specialized respiratory mechanism." The present writer confirms the head covering and records ridges containing blood vessels on the internal surface of the follicular wall.

Anti-thyroid compounds such as thiourea and related substances have been shown to affect the thyroid in all classes of vertebrates. The usual finding is an increase in the size of the gland and its cells and the production of a hypofunctioning organ. Studies have included many animals, among which are the following: rat (Astwood *et al.*, 1943; Mackenzie and Mackenzie, 1943; Hughes, 1944), monkey (McGinty and Wilson, 1949; Mixner *et al.*, 1944; Astwood *et al.*, 1944), reptiles (Ratzersdorfer *et al.*, 1949; Adams and Craig, 1949, 1950, 1951), amphibians (Gordon *et al.*, 1943, 1945b; Hughes and Astwood, 1944; Joel *et al.*, 1949; Adams and Craig, 1949), fishes (Goldsmith *et al.*, 1944; Nigrelli *et al.*, 1946; Lever *et al.*, 1949; Rasquin, 1949; Osborn, 1951; Chambers, 1951; Scott, 1953).

Goldsmith *et al.* (1944), reported hyperplasia of the thyroid in a hybrid strain of *Xiphophorous helleri* and *X. maculatus* after immersion of the fish in a thiourea solution. Their fish were inhibited in growth and failed to develop secondary sex characters. The studies of Rasquin (1949) on *Astynax mexicanus* and Scott (1953) on *Brachydanio rerio* substantiate the hyperplasia previously reported. Lever *et al.* (1949) investigated the effect of a short thiourea treatment upon the thyroid of *Lebistes reticulatus* and *Callionymus lyra*. They reported an increase in thyroid cell height as early as the first week. Fish in the present study showed extreme hyperplasia within eight days, but checks at shorter intervals were not made. In addition to the localized changes, metastasis to many areas of the body was induced.

In many animals, a hypofunctional state of the thyroid may be produced surgically or chemically by a number of goitrogenic compounds. The relationship between oxygen consumption and the metabolic rate is understood and known to be affected by the thyroid status in homoiothermal animals. Hypofunction leads to decreased oxygen consumption while hyperfunction results in the increase of oxygen consumption. Attempts to alter the oxygen uptake in fish by methods which have proven to be effective and reliable in other animals have yielded results that are contradictory. Drexler and Issekutz (1934) found that thyroxine in a 1 : 500 000 concentration failed to increase the metabolic rate of *Lebistes*. The work of Etkin *et al.* (1940) failed to reveal any increased metabolism after the feeding of desiccated thyroid tablets to goldfish for 9 weeks; the body weights were not changed even after treatment for 16 weeks. They interpreted the results as an absence of a metabolic effect of the thyroid in fish. Grobstein and Bellamy (1939), working with immature *Xiphophorus* (=*Platypoecilus*), produced an exaggerated exophthalmos, a change in body proportions and precocious sexual development. Smith and Everett in 1943 administered thyroxine and desiccated thyroid to new-born guppies for 50 to 90 days. They found no change in growth rate and the fish differentiated at the same time as the controls. Further, oxygen utilization was not different after a one-week exposure.

The administration of anti-thyroid drugs to fish has produced more consistent data. Goldsmith *et al.* (1944) considered the inhibition of growth and retarded

sexual development as (p. 133) "presumptive evidence that the thyroid gland is concerned with growth and development of sex characters of fishes." They concluded, "it appears that thiourea produces these effects by interfering with thyroid hormone production in much the same manner as reported for the mammal" (p. 133). Scott (1953) ascribed the retardation of growth in *Brachydanio* after thiourea administration to a hypofunctional thyroid. He stated (p. 59), "The evidence obtained from the observation of the glands indicated that such treatment produced a hypofunctional thyroid in the zebra fish." Nigrelli *et al.* (1946), working with *Lebistes reticulatus,* treated simultaneously with mammalian thyroid powder and thiourea, noted an increase in growth of these fish similar to that of their untreated controls and greater than that observed in their thiourea-treated fish. Osborn (1951) studied oxygen consumption by stonerollers (*Campostoma anomalum*) and bigmouth shiners (*Notropis dorsalis dorsalis*) immersed in thiouracil. Oxygen consumption was reduced about 20% with dosages of thiouracil that caused no mortality. The decrease was slight on the first day of treatment, but reached the 20% figure on the second day. A pronounced increase in oxygen consumption took place soon after replacing the fish in plain water. The increase was accomplished more slowly in fish treated for 22 days than in those treated for 9 to 11 days. The BMR is lowered in higher animals treated with goitrogenic compounds (Barker, 1949; Mackenzies, 1943; Leathem, 1945; Gordon *et al.,* 1946). Generally the decrease parallels that seen in surgically thyroidectomized animals. In view of the controversies concerning the fish thyroid, further study of the metabolic maintaining mechanism of these animals should be made.

The ovary of *Lebistes reticulatus* normally contains, after the presentation of young, a number of nearly mature ova (0.8–1.5 mm.) approximately equal to the number of young in previous broods. The number of the smaller and less developed ova (0.3–0.5 mm) is consistently larger than this. This finding indicates that a number of eggs undergo atresia. The thiourea-treated animals of groups 1D, 1W and 2W suffered a decrease in number of young in their fourth brood as well as a smaller than normal number of ova of the sizes mentioned above. Group 3W individuals, although presenting a fourth brood of normal number, possessed ovaries containing fewer than normal eggs of the 0.3–0.5 mm. group. These findings are evidence of a decreased ovarian function. The difference in the severity of reaction to thiourea between the groups is no doubt a reflection of the difference in time of exposure. Chambers (1951), Barrington and Matty (1952) and Scott (1953) also noted a gonad inhibition in fish after thiourea administration. It may be theorized that the decreased ovarian function is the result of a lowered production of a gonad-stimulating hormone elaborated by the pituitary, rather than being a direct action of the administered thiourea. Matthews (1939) has shown that gonadal function in the teleost *Fundulus* is dependent upon the presence of the pituitary. He recorded in 1939 that hypophysectomy results in lowered gonadal activity in that genus. It may well be that with the suppression of thyroid gland activity caused by thiourea, there is a great need for a rise in the titer of a pituitary thyroid-stimulating factor. The increased thyroid-stimulating factor production is accomplished by a sacrificing of the gonad-stimulating hormone to the level where the latter cannot maintain a normal functioning gonad. The present results are comparable to those reported to occur in rats. Barker (1949) noted a markedly

reduced fertility with altered estrous cycles after thiourea treatment. Decreases in size and weights of mice ovaries and uteri have been observed by Dalton *et al.* (1945). Jones, Delfs and Foote (1946) induced fetal resorption by thiouracil administration. They also stated that a decrease in the titer of gonadotropin occurred after prolonged treatment with the drug. The small size of the guppy makes hormone analysis of the blood a difficult, if not impossible, procedure at the present time.

It is well known that the secretory activity of the thyroid is regulated by an interaction and balance between that gland and the anterior lobe of the pituitary. A decrease in thyroid hormone output reflects itself in an increased production of the thyroid-stimulating hormone by the pituitary (Gordon *et al.*, 1945a; Grasso, 1946). A hypofunctioning thyroid may be produced by the administration of thiourea and related compounds. It has been found that the ability of the thyroid to take up iodine is depressed within one hour after the injection of anti-thyroid compounds into rats (Larson *et al.*, 1945). As a consequence of the decreased amount of circulating thyroid hormone and an increase in the stimulating factor, the thyroid is changed morphologically and physiologically. The thyroid tissue is a very labile one and soon demonstrates morphological alterations in an attempt to adjust its hormone output. Hyperemia and heightened epithelium are seen within 24 to 48 hours. Hyperplasia becomes pronounced in most forms within a week.

The scheme outlined in the preceding paragraphs, *viz.*, a decreased thyroid function followed by an increased circulating titer of thyroid-stimulating hormone and a decreased amount of a gonad-stimulating hormone, may well explain the interference with reproductive activities seen after thiourea immersion.

The thyroid tissue of all young of all treated groups gave evidence of great stimulation. Heightened cells, afollicular cell arrangement, masses of thyroid tissue without colloid, and metastasis to abnormal sites were found. Two main hypotheses may be offered to explain the stimulation. It may be argued that thiourea itself passed from the blood vessels of the maternal ovarian follicle wall to the embryo by way of the structures previously described. This compound, circulating within the embryo, suppressed the activity of its thyroid, resulting in an increased embryonic thyroid-stimulating hormone titer. The high level of the stimulating factor then caused morphological changes in the target organ. This hypothesis assumes fetal thyroid and pituitary function. The second possible explanation depends upon an increase in adult thyroid-stimulating factor (which must be granted in order to explain the adult thyroid changes) and the transfollicular transmission of this agent from the adult to the embryo. The maternally derived factor then stimulated the embryo thyroid. Valuable information on this question might be obtained if gravid females could be hypophysectomized in the latter part of the gestation period and subjected to thiourea starting at that time. If thiourea and an embryonic pituitary factor were responsible for the hyperplastic thyroid of the young noted in the present work, these same changes would follow this procedure. On the other hand, if the adult pituitary factor was the sole agent causing the hyperplasia the thyroid would not be changed. This method of attack assumes that the females could maintain and deliver their young after being deprived of the pituitary. Considering molecular size alone, other factors being equal, it would be easier for thiourea to pass from the adult to the embryo. The molecular weight of thiourea is 76.12. The

thyroid-stimulating hormone in a rather purified form obtained from sheep and beef pituitary glands possesses a molecular weight of approximately 10,000. Because of species differences, this finding cannot be carried over directly to the fish pituitary factor, but it does allow some speculation as to the size of the molecule. Direct placental transmission of anti-thyroid compounds has been shown to occur in the rat (Hughes, 1944; Williams, 1944; Goldsmith *et al.*, 1945) and in the mouse (Kaufman *et al.*, 1948).

Accepting the fact that the *Lebistes reticulatus* embryo is contained within an ovarian follicle and has no connection with the external environment except by way of the adult, one must concede that a transfollicular movement of material is possible and did occur in the present work. The use of the term ovoviviparous, in its strict sense, to describe the guppy is therefore open to question.

Summary

1. The adult thyroid of *Lebistes reticulatus* after exposure, by immersion, to a 0.04% thiourea solution displayed an extreme hyperplasia. An increase in follicle size and number and their establishment in abnormal areas took place. The heart, kidney, spleen, and intestines were invaded by the thyroid epithelial cells. The mean height of the follicular cells of the fish receiving the shortest thiourea treatment increased approximately 130%. With longer periods of exposure to thiourea, greater heights were produced.

2. The thyroid gland of the newborn of thiourea-treated females had undergone hyperplasia and metastasis during ontogeny. The thyroid became afollicular, lost colloid and possessed tall epithelial cells. Hearts, kidneys, spleens and intestines harbored the invading cells. A transfollicular transmission of an agent causing these changes took place.

3. *Lebistes reticulatus,* while not having an anatomical uterus, placenta or other means of attaching the developing embryo firmly to a part of the adult female reproductive system, does have maternal and embryonic modifications which permit an exchange of material. The species is therefore not a true ovoviviparous form.

4. The gametogenic function of the adult ovary was suppressed by thiourea administration as evidenced by abortions, decrease in brood size, longer brood intervals and smaller than normal number of ova in the gonad. This finding may be a reflection of a decreased gonad-stimulating hormone titer. The latter may be due to a shift to a greater thyroid-stimulating hormone production at the expense of a gonad-stimulating hormone by the pituitary.

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OSMOTIC STUDIES OF AMPHIBIAN EGGS. III. OVULATED EGGS

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In the two previous papers in this series (de Luque and Hunter, 1959; Hunter and de Luque, 1959) a study was made of the movement of water and cations into and out of the eggs of *Bufo marinus* and *Hyla labialis* with the emphasis on ovarian eggs. It was hoped that by making a similar study with fertilized and unfertilized eggs laid in water, it might be possible to find a basis to explain the apparent decrease in osmotic pressure of these eggs after they have left the ovary. These observations are included in the present paper.

Materials and Methods

As in the previous experiments, *Hyla labialis* were obtained locally and *Bufo marinus* were shipped to Bogotá by air. *Bufo* pituitaries suspended in 0.66% NaCl were injected to obtain ovulation. Since with these two species, sperm suspensions, obtained by removing the testes from a male, cannot be used to fertilize the eggs, the males also had to be injected. It has now been established (A. S. Hunter, unpublished data) that one can find *Hyla* with mature ova twice a year, associated with the rainy seasons. Pituitary injections at these two seasons usually result in fertilized eggs. However, in the case of *Bufo* nothing is known about their normal season in Colombia for ovulation, and consequently fertilized eggs were obtained from this species only occasionally.

As previously described, the jelly layer was removed from the eggs with watch-maker's forceps and the eggs were suspended in various NaCl and KCl solutions. Since volume changes have previously been reported, only a few of these measurements were made. Water and Na and K content were measured as before. A new attempt was made to measure non-solvent water using a distribution technique (*cf.* Parpart and Shull, 1935). The quantity of glycerol was determined by the method of Bailey (1959).

Results

Bufo ovarian eggs. In the first paper of this series it was suggested that *Bufo* ovarian eggs separated from their follicular layers swelled less than these eggs plus their follicular layers. In those experiments volume changes of eggs plus layers were measured after a short exposure and after a longer exposure to solutions with different osmotic pressures. Similar measurements using eggs without layers were made only after short exposures to the solutions since such eggs rapidly become aspherical. Since the apparent difference in volume changes

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between eggs with and without their follicular layers could have resulted simply from a difference in time of exposure, these experiments were repeated with observations at the end of an hour and at the end of 24 hours. At the end of an hour's exposure to solutions of NaCl (0.75–0.55%) the same small volume changes were observed with both types of eggs. This is similar to the changes previously reported (Fig. 2, p. 462 and the open symbols in Fig. 1, p. 461). At the end of 24 hours, the eggs without follicular layers swelled appreciably in all of the solutions (*cf.* closed symbols in Fig. 1, p. 461) with larger volume changes occurring in more hypotonic solutions. Measurements of Na and K content of these eggs after 24 hours in these solutions also indicated that Na and K could move across the cell membrane. It can be concluded, then, that water and salts can move slowly into ovarian eggs and also into the cells forming their follicular layers.

TABLE I

The amount of Na or K present in jelly following suspension in solutions of NaCl or KCl

Number of experiments	Na—meq./mm.^{3} X10^6					
	0.60% NaCl		0.38% NaCl		0.20% NaCl	
	Observed	Calculated	Observed	Calculated	Observed	Calculated
8	9.9	10.2	8.0	6.5	5.6	3.4
	K—meq./mm.^{3} X10^6					
	0.60% KCl		0.38% KCl		0.20% KCl	
7	7.6	8.0	5.1	5.1	2.9	2.7

Ovulated eggs. In all of the experiments made using eggs laid in water, whether fertilized or not, interpretation of the data becomes difficult because of the presence of a layer of jelly of varying thickness surrounding the eggs. The majority of the jelly was removed but it is almost impossible to obtain a completely clean egg on which to experiment. Moreover, if all of the jelly is removed the eggs do not develop normally (a higher percentage of exogastrulae results). For this reason, studies had to be made of isolated jelly in order to determine its contribution to the results obtained from eggs with a small jelly layer.

Since it was difficult to obtain sexually active males and females of *Bufo* simultaneously, only a few samples of *Bufo* fertilized eggs were studied. Because of the theoretical importance of a possible change in the amount of non-solvent water in the ovulated eggs (as compared to ovarian eggs) all of the samples of fertilized *Bufo* eggs were used for these studies, which unfortunately did not give repeatable results. The data on water, Na and K content of isolated jelly and of laid (unfertilized) *Bufo* eggs and of fertilized *Hyla* eggs follow.

Jelly. The jelly will be considered first, since it is involved in all of the other determinations. The difference between wet and dry weight showed that at least

TABLE II

Changes in water content of unfertilized eggs of Bufo marinus placed in solutions of NaCl of different osmotic pressures

Bufo no.	0.60% NaCl		0.38% NaCl		0.20% NaCl		Water	
	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg
1	87	4.96	90	6.02	87	4.97	95	13.39
2	88	6.16	86	5.09	89	6.69		
3	95	5.37	96	9.50	95	6.47		
Av.	90	5.50	91	6.87	90	6.04		

TABLE III

Changes in salt content (meq./mg. $\times 10^6$) of unfertilized eggs of Bufo marinus placed in solutions of NaCl of different osmotic pressures

Bufo no.	0.60% NaCl		0.38% NaCl		0.20% NaCl		Water	
	Na	K	Na	K	Na	K	Na	K
1	24.8	14.0	21.8	14.8	19.0	14.0	7.3	15.0
2	33.4	16.0	27.6	16.4	18.6	14.0		
3	25.8	4.8	27.7	4.5	13.0	3.9		
Av.	28.0	11.6	25.7	11.9	16.9	10.6		

TABLE IV

Changes in water content of unfertilized eggs of Bufo marinus placed in solutions of KCl of different osmotic pressures

Bufo no.	0.60% KCl		0.38% KCl		0.20% KCl	
	%	mg./egg	%	mg./egg	%	mg./egg
1	89	5.42	88	5.96	90	6.91
2	82	4.44	88	6.68	87	6.24
3	87	3.09	88	3.77	93	6.42
Av.	86	4.32	88	5.47	70	6.52

TABLE V

Changes in salt content (meq./mg. $\times 10^6$) of unfertilized eggs of Bufo marinus placed in solutions of KCl of different osmotic pressures

Bufo no.	0.60% KCl		0.38% KCl		0.20% KCl	
	Na	K	Na	K	Na	K
1	13.5	45.0	6.8	29.2	7.7	39.2
2	7.4	35.5	8.2	23.2	5.8	20.2
3	8.0	59.9	3.1	23.3	4.3	28.7
Av.	9.6	46.8	6.0	25.2	5.9	29.4

TABLE VI

Changes in water content of fertilized eggs of Hyla labialis placed in solutions of NaCl of different osmotic pressures

Hyla no.	0.60% NaCl		0.50% NaCl		0.38% NaCl		0.20% NaCl		Water	
	%	mg./egg	%	mg/.egg	%	mg./egg	%	mg./egg	%	mg./egg
1	75	1.93	78	2.01	79	2.30	81	3.11	87	4.24
2	65	1.43	77	1.82	72	1.45	75	1.60	90	5.93
3	80	1.92			82	2.96	83	2.40		
4	71	1.52					82	3.06		
Av.	76	1.70	78	1.92	78	2.24	80	2.54	89	5.09

99.5% of the jelly of both types of eggs is water. Solvent water measurements, which in the case of jelly are probably valid, showed that 100% of the water in the jelly is solvent. In 6 determinations on *Hyla* jelly, values between 98 and 144% were obtained.

Hyla jelly was placed in NaCl and KCl solutions of different concentrations. After one hour the Na and K content of the jelly was determined. In Table I a comparison is made between the amount of Na or K present in the jelly after one hour in the various solutions and the theoretical amount expected with diffusion equilibrium. In solutions of NaCl, no K was present in the jelly and vice versa. From this it can be concluded that normally there is little, if any, Na or K in jelly.

It can readily be seen that in all of the six solutions there is good agreement between the calculated and observed values. It can be concluded that in solutions of NaCl and KCl the jelly reaches diffusion equilibrium rather rapidly.

To calculate the volume of jelly surrounding each egg, measurements were made in water of the diameter of the egg and of the egg plus jelly. In one series of experiments the average volume of an egg was found to be 1.98 mm.^{3} and the volume of the egg plus jelly, 5.60 mm.^{3}. Even when extreme care was used to remove as much jelly as possible without injuring the eggs these volumes were 1.65 mm.^{3} for an egg and 2.86 mm.^{3} for egg plus jelly. Similar measurements of eggs in 0.38% NaCl gave an average value of 1.03 mm.^{3} for the volume of an egg and 2.11 mm.^{3} for the volume of egg plus jelly. The difference between these

TABLE VII

Changes in salt content (meq./mg. $\times$ 10^3) of fertilized eggs of Hyla labialis placed in solutions of NaCl of different osmotic pressures

Hyla no.	0.60% NaCl		0.50% NaCl		0.38% NaCl		0.20% NaCl		Water	
	Na	K	Na	K	Na	K	Na	K	Na	K
1	15.8	18.0	10.6	13.1	8.1	13.2	10.0	13.5	4.1	11.9
2	17.2	14.0	10.8	11.2	11.4	11.8	4.5	8.5	4.8	12.9
3	19.6	10.5			16.2	14.0	11.7	8.7		
4	13.7	10.1					16.0	14.7		
Av.	16.6	13.2	10.7	12.2	11.9	13.0	10.7	11.4	4.5	12.4

TABLE VIII

Changes in water content of fertilized eggs of Hyla labialis placed in solutions of KCl of different osomotic pressures

Hyla no.	0.60% KCl		0.38% KCl		0.20% KCl		Water	
	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg
1	81	2.42	84	2.93	80	2.53	83	2.86
2	61	1.73	72	1.91	63	1.45	84	4.08
3	75	2.17	77	2.46	73	2.20	88	5.64
4	64	1.26	67	1.44	72	2.05	85	4.09
5	72	2.05	77	2.83	60	1.22	83	4.60
Av.	71	1.93	75	2.31	70	1.89	85	4.25

values obtained in water and in 0.38% NaCl suggests that in water both the egg and the jelly swell but much more water is taken up by the jelly. It can be seen that in all of the subsequent experiments to be reported the volume of the jelly is larger than the volume of the eggs.

Bufo, unfertilized eggs. Tables II–V show the changes in water and salt content of unfertilized *Bufo* eggs after having been in various NaCl and KCl solutions during an hour. Since these eggs are always very irregular in form, volume measurements were not made.

Hyla, fertilized eggs. Similar data for *Hyla* fertilized eggs are presented in Tables VI–IX.

ANALYSIS OF RESULTS

Because of the nature of the above experiments, various assumptions have to be made in trying to interpret the data. It is quite possible to change one's assumption and arrive at a completely different conclusion. The analysis which follows, then, represents one possible interpretation of the data. The only point the authors want to make is that the data are not inconsistent with these suggestions.

In general, a comparison of the above data with those previously reported shows that all of the changes are less with ovarian eggs. From the data in Table X it can be seen that the volume of unfertilized eggs (plus jelly) of *Bufo* is much

TABLE IX

Changes in salt content (meq./mg. $\times 10^5$) of fertilized eggs of Hyla labialis placed in solutions of KCl of different osmotic pressures

Hyla no.	0.60% KCl		0.38% KCl		0.20% KCl		Water	
	Na	K	Na	K	Na	K	Na	K
1	7.9	28.6	8.8	18.9	7.5	15.5	7.8	15.0
2	5.9	15.8	3.7	10.5	5.1	28.0	4.6	7.2
3	5.6	14.2	5.5	12.8	2.9	25.2	5.5	8.0
4	6.0	12.8	6.0	12.6	4.7	25.1	5.1	8.4
5	5.0	13.8	4.7	13.1	3.2	25.6	4.7	8.2
Av.	6.1	17.0	5.7	13.6	4.7	23.9	5.5	9.4

larger than that of ovarian eggs. The unfertilized eggs also have more Na and K. Fertilized *Hyla* eggs (plus jelly) are much larger than ovarian eggs and in NaCl solutions they have more Na and K. The presence of the jelly obviously could contribute to this difference.

One interesting point of comparison is that in spite of the differences in quantity and percentage of water in the eggs in the different solutions (Tables II, IV, VI, and VIII) the dry weight per egg, as calculated from these data, is always essentially the same, as would be expected. The dry weight of a *Bufo* ovarian egg as previously published was 0.59 mg. The unfertilized eggs in NaCl and KCl solutions give values of 0.61–0.75 mg./egg. The published figure for *Hyla* ovarian eggs is 0.65 mg./egg. In the present experiments with fertilized eggs the values are 0.54–0.81 mg./egg. Considering the difference in size of eggs from different females it seems reasonable to say that the eggs neither gain nor lose considerable quantities of substances included in the dry weight when they leave the ovary.

It is obvious from the measurements mentioned earlier of the volume of an egg and the volume of its jelly that a large part of the volume changes in Tables II, IV, VI, and VIII may be due to changes in the amount of water in the jelly. To try to determine whether or not water enters or leaves the egg let us consider that 0.38% salt solution has one unit of osmotic pressure and that the volume of the water in the egg plus jelly is 1. The expected volume of water in any other solution can be calculated using the formula PV = constant. Table XI compares these calculated values with the actual changes in the volumes of the eggs.

TABLE X

A comparison of water (mg./egg) and salt content (meq./mg. × 10^5) of ovarian and laid eggs in isotonic solutions (0.66% and 0.38%, respectively)

Type of egg	NaCl			KCl		
	Water	Na	K	Water	Na	K
Bufo, ovarian	1.17	12.2	7.8	0.93	2.6	12.9
Bufo, non-fertilized	6.87	25.7	11.9	5.47	6.0	25.5
Hyla, ovarian	0.64	7.2	3.1	1.47	5.8	15.7
Hyla, fertilized	2.24	11.9	13.0	2.31	5.7	13.7

With the exception of the first value, the changes in the volume of water are always less than would be expected. That is, in hypertonic solutions the eggs shrank less and in hypotonic solutions they swelled less than the calculated values. An average of the per cent deviation of the last 6 values gives a figure of 28%. Using the figures previously given (egg plus jelly, 2.11 mm.^{3}; egg, 1.03 mm.^{3}) 28% of 2.11 mm.^{3} gave a value very similar to the quantity of water in an egg with a volume of 1.03 mm.^{3}. Using the other measurements (egg plus jelly, 5.60 mm.^{3}; egg, 1.98 mm.^{3}) a similar calculation leads to the same conclusion. This would suggest that at least the major portion of the change in water content occurred in the jelly. Tentatively, then, let us suggest that these studies of changes in water content might be interpreted as indicating a decreased rate of movement of water across the membrane of an egg after it has left the ovary.

TABLE XI

A comparison of the theoretical and observed volume changes in water content

Egg	Solution	Water content		
		Calculated volume	Observed volume	% difference
Bufo	0.60% NaCl	0.63	0.62	2
	0.60% KCl	0.63	0.79	25
	0.20% KCl	1.90	1.20	37
Hyla	0.60% NaCl	0.63	0.76	21
	0.50% NaCl	0.76	0.85	12
	0.20% NaCl	1.90	1.13	41
	0.60% KCl	0.63	0.84	33

In Table XII an attempt was made to determine how much of the increase in salt in the egg could be attributed to salt dissolved in the jelly. Two methods were used to calculate the *Hyla* data. The volume of the egg was considered to be 1.03 mm.^{3} (obtained from the measurements previously given) or 1.44 mm.^{3} (obtained by adding the dry weight to the amount of water in an ovarian egg). Only the latter method (volume = 1.23 mm.^{3}) was used in calculating the *Bufo* data. It was assumed that this volume did not change in the different solutions. (Probably this assumption is not completely correct but the error introduced would not change the argument.) This value was subtracted from the total volume of water actually measured in the various solutions (Tables II, IV, VI, and VIII) to give a minimum value for the amount of water in the jelly. The volume of jelly water was then multiplied by the calculated milliequivalents of Na or K in the different solutions to give a theoretical value for the amount of Na or K in the water in the jelly, assuming diffusion equilibrium. These figures can then be compared with the total amount of Na or K per egg calculated on the basis of 0.65 mg. per egg as a dry weight of *Hyla* eggs and 0.59 mg. for *Bufo* eggs.

In the case of *Bufo* eggs, comparing the last two columns, with the exception of 0.20% KCl (these data are inconsistent in every respect), the increase in measured amounts of Na and K could have been due completely to the solution

TABLE XII

A comparison between calculated values of Na and K in the jelly layer and observed values in eggs plus jelly

Solution	Ion	Calculated quantity in *Hyla* jelly		Obs. *Hyla*	Calculated quantity in *Bufo* jelly (1.23 mm.^{3})	Obs. *Bufo*
		(1.03 mm.^{3})	(1.44 mm.^{3})			
0.60% NaCl	Na	6.9	2.7	10.8	44.0	16.5
0.38% NaCl	Na	7.9	5.2	7.7	36.7	15.2
0.20% NaCl	Na	5.1	3.7	7.0	16.3	10.0
0.60% KCl	K	7.2	3.9	11.0	24.8	27.6
0.38% KCl	K	6.5	4.2	8.8	21.6	14.9
0.20% KCl	K	2.3	1.2	15.5	14.2	17.3

of these ions in the jelly. In the case of *Hyla* eggs, the same conclusion could be reached. This suggestion could also explain the observation that in the present experiments there is more salt in the eggs plus jelly in more concentrated salt solutions, while with the ovarian eggs the amount of salt in the eggs was essentially the same regardless of the concentration of the surrounding solution.

Another method to analyze the data to test the same point follows. If we assume that the concentration of Na or K in the jelly is the same as in the surrounding solution, and if we further assume that the amount of Na or K in the egg is constant, three simultaneous equations can be solved for each series of experiments to determine the amount of Na or K in the eggs. If we let a = volume of jelly in mm.^{3} and b = milliequivalents of Na (or K) in the egg, then for *Bufo* eggs in NaCl solutions:

$$10.3 \times 10^{-5}a + b = 19.1 \times 10^{-5}$$
$$6.4 \times 10^{-5}a + b = 17.5 \times 10^{-5}$$
$$3.4 \times 10^{-5}a + b = 11.5 \times 10^{-5}$$

With these three equations an average value of 1.17 mm.^{3} for the volume of the jelly was obtained. Using this value, the amounts of Na in the *Bufo* eggs in the three NaCl solutions were 7.0, 10.1 and 7.5×10^{-5} meq./mg. These values are quite similar to those previously obtained with ovarian eggs. Making similar calculations for *Bufo* eggs in KCl solutions and for *Hyla* eggs in NaCl and in KCl solutions, the values for Na or K in the eggs in these experiments are similar to, or less than, the amount previously reported in ovarian eggs.

The above calculations would suggest that salts as well as water enter and leave ovarian eggs more rapidly than they enter and leave eggs that have left the ovary.

Another calculation which is of interest is that previously made with ovarian eggs (Hunter and de Luque, last line, p. 476). From the data in Table II, the dry weights of the eggs in each of the three solutions of NaCl can be calculated. Multiplying these dry weights by the values of Na and K in Table III, the total milliequivalents of cations per cell can be calculated. Dividing this by the amount of water per cell, the concentration (in milliequivalents of Na plus K per liter) can be calculated. Values of 44, 37 and 31 were obtained with these data. These values are to be compared with 140 previously calculated for ovarian eggs. Similar calculations with *Bufo* eggs in KCl solutions and *Hyla* eggs in NaCl and KCl solutions yielded values considerably lower than for ovarian eggs. Although it is difficult to assess the effect of the jelly in these calculations, one might conclude that the osmotic pressure of eggs that have left the ovary is less than the osmotic pressure of ovarian eggs.

Discussion

The various measurements of volume changes of ovarian eggs, unfertilized and fertilized eggs in solutions with different osmotic pressures have shown that water can move into or out of the three different types of eggs. The studies of Na and K content also suggest that these cations can move across the membranes of the three types of eggs. One analysis of the present data leads us to the conclusion that a decrease in permeability to water and salts in eggs that have left the ovaries is not inconsistent with the data. As was mentioned in the review of the literature

in the first paper of this series, similar suggestions have previously been made, based on studies of various eggs.

If the above suggestion is correct, it could explain how an egg laid in water could survive without losing all of its salts and without swelling indefinitely due to the entrance of water. However, it does not explain the apparent decrease in osmotic pressure in the eggs when they are laid.

The present data do not support the idea that the decrease in osmotic pressure of the eggs after they have left the ovary results from the loss of salts or from the entrance of a large quantity of water. On the contrary, the data in Table XIII suggest that there is an increase in the amount of K in the eggs that have left the ovary. The values of t (calculated by the Method of Fischer) indicate that there is no difference in the amount of Na in *Hyla* ovarian and fertilized eggs but there is a difference in K in these eggs significant at the 2% level. There is no significant difference in the amount of Na in *Bufo* ovarian and unfertilized eggs but a highly significant difference in the amount of K in these eggs.

In making the salt analyses of the egg homogenates, the proteins are precipitated with trichloracetic acid. This means that whatever quantities of Na and/or

TABLE XIII

A comparison of the Na and K content of ovarian and non-ovarian eggs of Hyla labialis and of Bufo marinus

	meq./mg. $\times 10^5$		t
	Ovarian	Ovulated	
Hyla, Na content	4.4 ± 0.20	5.5 ± 1.22	0.32
Hyla, K content	5.4 ± 0.03	12.4 ± 0.95	4.10
Bufo, Na content	6.8 ± 0.32	7.2 ± 0.29	3.12
Bufo, K content	8.3 ± 0.25	12.3 ± 0.31	72.0

K are bound to the proteins of the egg are not included in the Na and K measurements. If one postulates: (1) that in the ovarian eggs some K is bound to proteins, and (2) that on leaving the ovary there is a change in the proteins which decreases their capacity to bind K, the observed increase in K could be explained. If this is true, one might also postulate that the amount of water bound to proteins decreases. Unfortunately, with the methods available in this laboratory, satisfactory values of non-solvent water could not be obtained. Using the glycerol-distribution technique with fertilized eggs of *Hyla*, the amount of glycerol that disappeared from the solution increased more or less indefinitely with time. The possibility that these eggs are metabolizing glycerol is being investigated. Because of the importance of determining whether or not there is a change in solvent water when the eggs leave the ovary, one of the authors (O. de L.) will continue these studies in another laboratory using isotopic techniques.

SUMMARY

1. Studies of eggs of *Bufo marinus* and of *Hyla labialis* that have left the ovary indicate that there is no large change in the total amount of water nor of Na and K.

2. A possible interpretation of the data is that after the eggs have left the ovary there is a decrease in their permeability to water and to Na and K.

3. A statistically significantly higher quantity of K in these eggs might suggest a change in the binding-capacity of the egg proteins.

4. If such a change does occur, there might be a decrease in the amount of water bound to the proteins which might explain the lower osmotic pressure of eggs that have left the ovary. Attempts to obtain experimental data to support this hypothesis failed for technical reasons.

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PREPARATION AND USES OF INSECT HEMOCYTE MONOLAYERS IN VITRO[1]

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Repeated subculturing of rapidly proliferating insect cells has not as yet been achieved, and to date no insect cell strain is available (Day and Grace, 1959; Martignoni, 1960). On one occasion a series of tissue subcultures from *Callosamia promethea* (Drury) suggested adaptation to a culture medium (Grace, 1958), but a cell strain was not established. This lack of a continuous cell strain may be responsible for the relatively slow progress in insect virology as compared to the rapid advances in vertebrate virology.

Explantation techniques have been adopted frequently by insect morphologists. However, for biochemical and virological purposes the use of cell suspensions is the most appropriate, and a number of investigations in this direction have been undertaken (St. Amand and Tipton, 1954; Martignoni, Zitcer and Wagner, 1958; Liu, Hsia and Kao, 1958; Aizawa and Vago, 1959). Recently we have developed a method for the preparation of monolayer cultures using insect hemocytes. Although these monolayers have the few disadvantages of primary cultures, they can be prepared easily and the cells are not subjected to treatment with enzymes or chelating agents. Insect blood is a good source of cells. Between 700,000 and 800,000 cells per larva are routinely obtained from the variegated cutworm, *Peridroma saucia* (Hübner) [= *P. margaritosa* (Haworth)], using the technique described in this paper. In comparison, the embryo of the green-striped grasshopper, *Chortophaga viridifasciata* (De Geer), produced about 13,000 cells after hyaluronidase and trypsin digestion (St. Amand and Tipton, 1954), and approximately 160,000 cells can be obtained from the three thoracic segments of a mature larva of *P. saucia* after treatment with snail hepatopancreas extract (Martignoni, Zitcer and Wagner, 1958).

As vertebrate leukocytes have been used successfully for the propagation of viruses (Dunne, Luedke and Hokanson, 1958), and since some insect viruses are known to multiply in hemocytes, *in vivo*, an attempt to prepare hemocyte monolayers for the propagation of insect viruses seemed justified.

Methods

Hemocyte source

Hemocytes were obtained from 2- to 4-day-old sixth instar larvae (last larval instar) of *Peridroma saucia* (Hübner) [= *P. margaritosa* (Haworth)] mass-

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produced according to methods described by Finney (1958). The mean weight of these larvae was 923 mg.

Balanced salt solution (BSS)

Composition. See Table I.

Preparation. This balanced salt solution (BSS) was prepared with extremely high-resistance water obtained by passage of distilled water through a 30 × 1¾-inch column of mixed-bed analytical grade ion exchange resin (AG 501-X8, D, 50–100 mesh; Bio Rad Laboratories; Richmond, California) and storage in polyethylene bottles. One hundred-ml. amounts of saline were prepared as follows: the salts, orthophosphorous acid, and l-ascorbic acid were dissolved in 90 ml. water, and the pH adjusted to 6.45 with 1 *M* NaOH. Distilled, de-ionized water was added to complete the volume of 100 ml., and the solution was sterilized by filtration (Millipore filter, type HA). This saline was preserved in the refrigerator, in rubber-stoppered 100-ml. bottles, for about one week; after

TABLE I

Composition of the balanced salt solution (BSS)

Compound	Grams/liter	Molarity
KCl	6.262	0.084
$CaCl_2$	2.553	0.023
$MgCl_2 \cdot 6H_2O$	3.660	0.018
$H_2(HPO_3)$	1.149	0.014
NaOH	1.068	0.027
l-ascobic acid	1.000	0.006

mole ratio Na/K	0.3
mole ratio K/Ca	3.7
pH	6.45
osmotic pressure (moles NaCl)	0.160

this period much of the ascorbic acid was oxidized and the solution was discarded. For a large-scale operation, it is advisable to have on hand large volumes of NaOH-neutralized solution of salts and orthophosphorous acid, without ascorbic acid. Whenever small volumes of fresh solution are needed (50 to 100 ml.) l-ascorbic acid is added to this stock solution, then neutralized (to pH 6.45), and the saline filtered through a Millipore membrane. The ascorbic acid-free stock solution can be used for the preparation of the culture medium, too (see below).

Although phosphate buffers have been used repeatedly in salines and culture media for insects (*e.g.* Trager, 1935; Ludwig, Tefft and Suchyta, 1957), their use limits the Ca and Mg levels in the solutions. The solution described here is buffered with sodium hydrogen phosphite, which has one considerable advantage when compared to phosphate buffers: the concentration of calcium and magnesium ions which can exist with phosphite in solution, without precipitation, is much greater than that with phosphate (Robertson and Boyer, 1956). This advantage is particularly important in insect tissue culture, as the salines often must contain large amounts of the two cations. A detailed comparison of the solubility of phosphite and phosphate salts has been presented by Robertson and Boyer.

Furthermore, the buffering capacity of phosphite is highest in the vicinity of pH 6.5, the blood pH of sixth-instar *P. saucia*. The approximate pK_2 of phosphite is 6.5. Orthophosphite is known to be nontoxic to cultured mammalian cells.

The blood pH of mature sixth-instar larvae of *P. saucia* was measured in humidified gas mixtures with various proportions of carbon dioxide. Equilibration with CO_2 was necessary, as there is a rapid loss of the gas from insect blood in air (Buck and Friedman, 1958). The pH measurements were made by using a one-drop electrode assembly with a silver chloride reference electrode (Fig. 1).

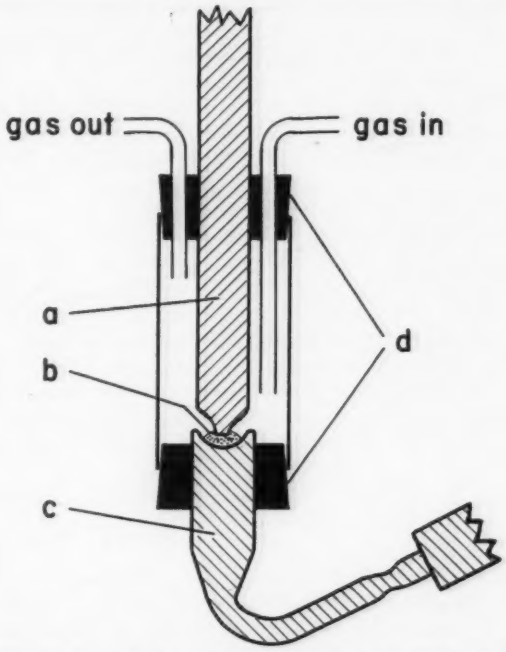


FIGURE 1. Electrode assembly for the measurement of the blood pH in an equilibration chamber: a, silver chloride reference electrode; b, blood; c, one-drop glass electrode; d, rubber stoppers used as closure of glass cylinder.

To obtain blood, an incision was made on one proleg of a larva, and bleeding followed immediately. With sufficient practice, two operators were making the first pH measurement within 10 seconds. Each determination was made on the blood obtained from a single larva, and each curve involves 9 to 15 larvae. The results are reported graphically in Figure 2. The least shift in pH was observed with 5% CO_2, with only a slight drift towards lower values. The 45 values obtained at the first measurement (5–15 seconds after blood extraction) formed a statistically homogeneous population: the mean was pH 6.47, with a standard error of 0.012.

As a basis for establishing the ion ratios in the BSS, the ionic content of four samples of pooled blood plasma (10 to 20 individuals) from sixth-instar larvae of *P. saucia* was determined by flame photometry. The mean contents were the following: Na, 10.3 meq. per liter; K, 33.5 meq. per liter; and Ca, 18.6 meq. per liter. The mean mole ratios were 0.3 (Na/K) and 3.7 (K/Ca). These mole ratios are well in agreement with those of other related phytophagous species

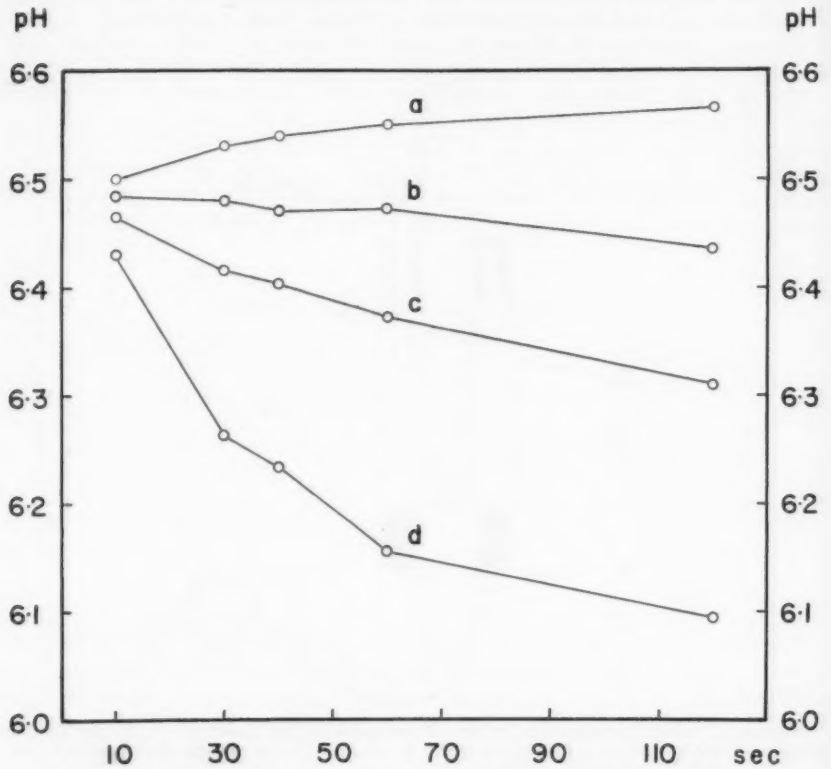


FIGURE 2. Results of pH measurements on whole blood of 2–4-day-old sixth-instar larvae of *Peridroma saucia* (Hübner), in an equilibration chamber, under a flow of: a, air; b, 5% CO_2 and 95% N_2; c, 20% CO_2 and 80% N_2; d, 100% CO_2. Time: seconds from start of bleeding.

(see the comprehensive compilation of Buck, 1953). It is now well established that these ionic ratios (particularly K/Ca) are very important in the formulation of physiologic solutions for insects (Ludwig, Tracey and Burns, 1957).

The BSS is isotonic with the blood of sixth-instar larvae of *P. saucia*. The blood osmotic pressure was determined by the method of Gross (1954). This method permits determinations on samples of less than 0.01 ml. volume to an

accuracy of 0.02° C. Twenty-one sixth-instar larvae were used, and the determinations were replicated two or three times for each larva. The mean osmotic pressure computed from this sample and expressed in m*M* NaCl was 155 (range: 138 to 176), with a standard error of 2.6 m*M*.

One of the important characteristics of the saline described here is that it is poised at a low oxidation-reduction potential (E_h around + 80 mv), with the addition of l-ascorbic acid (molarity: 0.006). Phenylthiourea has been recommended as a tyrosinase inhibitor in insect tissue culture (Schmidt and Williams, 1953), but we found this substance to affect cell cultures adversely. This confirms previous observations by Wyatt (1956), who reported toxic effects of this substance, or an impurity in it, in tissue cultures of *Bombyx mori* (Linnaeus). The mechanisms of the adverse effects of phenylthiourea are not well known, but it is possible that this substance may act also on other vital oxidative reactions: DuBois and Erway (1946) demonstrated that rat lung succinic dehydrogenase was considerably inhibited by thiourea (100% inhibition) and by phenylthiourea (94% inhibition). Jones and Wilson (1959) found that phenylthiourea had a pronounced depressant action on the rate of oxygen consumption of final instar larvae of *Samia cynthia* (Drury). This depressant action may be exerted on parts of the respiratory chain, according to a number of investigators. Ascorbic acid does not specifically inhibit tyrosinase, but maintains a low oxidation-reduction potential in the saline (approaching the situation in the larval hemocoel), sufficiently so to avoid immediate melanosis in cell cultures. The useful concentration of ascorbic acid (6 m*M*) was determined by recording the reactions of the cells and blood plasma to varying amounts of the substance, from 0.23 m*M* to 0.23 *M* per liter. Since l-ascorbic acid undergoes oxidation, the solution must be used fresh (from one to five days old), and stored at 4° C. While we used ascorbic acid, it is possible that other oxidation-reduction systems may work as well.

Maintenance medium (26C)

Composition. See Table II.

Preparation. Medium 26C was prepared with stock solutions: 100 × concentrates of the L-amino acids and of the vitamins are available commercially, and were routinely used in our laboratory. A 100-ml. volume of medium 26C was prepared as follows: (1) place in a beaker 90 ml. of ascorbic acid-free, buffered (pH 6.45) BSS; (2) add 1 ml. each of 100 × concentrates of the amino acids and vitamins; usually L-glutamine is stored as a separate 100 × concentrate; (3) add 1 ml. of 100 × concentrate of penicillin and streptomycin; (4) add and let dissolve 70 mg. glucose, 40 mg. fructose, and 40 mg. trehalose; (5) add and let dissolve 100 mg. l-ascorbic acid and carefully neutralize, under constant stirring, using molar NaOH solution, to pH 6.45; about 1 ml. will be required; (6) bring volume up to 100 ml. with BSS; (7) sterilize by filtration (Millipore filter, type HA); store at 4° C.; do not use if older than 5 days.

Fetal bovine serum gave better results than human, lamb, and horse sera. However, we have not completed yet a systematic investigation of a number of serous fluids. In this connection, it is worth mentioning a report by Gessard (1901), who found that calf and rabbit sera have tyrosinase-inhibiting properties.

TABLE II

Composition of the maintenance medium (26C)

Components	Concentration
Inorganic compounds and l-ascorbic acid	As in BSS described above
13 L-amino acids HeLa	As in Eagle (1955)
9 vitamins	As in Eagle (1959)
3 carbohydrates	As in Wyatt (1956), with d(+) trehalose instead of sucrose
Antibiotics	
Penicillin G	200 units/ml.
Streptomycin sulfate	100 μg./ml.

This medium was supplemented with 15% homologous insect blood plasma and 15% fetal bovine serum.

Gas mixture

Cultures prepared with medium described above were held in a gas mixture of 96% (by volume) nitrogen (minimum purity 99.996%), 3% carbon dioxide (minimum purity 99.95%), and 1% oxygen (minimum purity 99.5%). The three gases were mixed after measuring the rate of flow of each in Matheson Universal Flowmeters. The cultures were kept in a specially adapted jar (capacity: 9 liters), provided with a double phase manometer: after evacuation to 15 inches of mercury the jar was refilled with nitrogen (to lower the oxygen partial pressure), then the flow of the N_2-CO_2-O_2 mixture was maintained at the rate of 600 ml. per minute for 2–3 hours. The low level of oxygen was selected in order to inhibit phenol oxidase activity. A series of tests performed with blood of sixth-instar larvae of *P. saucia* showed that the period from blood extraction to onset of melanosis was prolonged by a factor of 2 or more only when the oxygen content of the gas mixture bathing the fluid was decreased from normal atmospheric level to less than 3%. No melanosis occurred in pure nitrogen. Tyrosinase in insect plasma can be inactivated by heat treatment (Wyatt, 1956); however, satisfactory culture of hemocytes of *P. saucia* is not possible even in the presence of inactivated homologous plasma, unless the oxidation-reduction potential of the culture medium is maintained at a low level by the addition of reducing agents and by exclusion of excess oxygen. We have observed cell melanosis in our cultures even in total absence of homologous plasma. As reported by Kawase (1960), in the case of mature silkworm larvae, hemocytes have a limited, but fairly constant tyrosinase content.

There is some indication in the literature that certain insect tissues may be considered microaerophilic: the hemocytes of *P. saucia* may be placed in this category. Schneiderman, Ketchel and Williams (1953) indicated that spermatogenesis in cultures of spermatocytal cysts of *Hyalophora cecropia* (Linnaeus), in presence of phenylthiourea, is not inhibited by reductions of the oxygen tension from normal atmospheric partial pressure to about 1% of atmosphere. Inhibition was observed below 1%. Similarly, Harvey and Williams (1958) observed that the heartbeat of diapausing pupae of the same species was independent of external oxygen tensions from normal to 0.5% of atmosphere. Oxygen tension seems to

have an effect also on the rate of growth of some mammalian cells. It is worth mentioning that Cooper, Burt and Wilson (1958) found that the liquid-phase oxygen levels for fastest growth of a rabbit kidney cell line were considerably less than air equilibrium values, and as low as 14% oxygen by volume.

Temperature

All cultures were held at 25° C., ± 0.5° C., in darkness.

Preparation and maintenance of cultures

The larvae of *P. saucia* were anesthetized with ethyl ether for about ten minutes, surface sterilized with 0.2–0.4% aqueous solutions of Hyamine 10-X for 5 minutes, with agitation (Martignoni and Milstead, 1960), and then allowed to dry between folded sterile paper towels. The larvae were placed in the germicide solution in the general laboratory area, but the transfer from germicide to the sterile towels was best performed in the culture room. Fifteen to twenty larvae could be handled as one group. It is not advisable to use more than this number at one time, since the effects of the ether anesthesia might end before the last larva is bled, and regurgitation, a common aftereffect of anesthesia in these larvae, may occur. About two minutes were allowed for drainage of the germicide. During this time, 3 to 4 ml. of culture medium were placed in a 12-ml. conical graduated tube, covered with an inverted glass beaker: the exact amount was noted. Each larva was lifted with forceps and carefully placed on the forefinger of the left hand (the operator was right-handed), taking care that the ventral integument of the larva did not come in contact with the skin of the operator. Thumb and middle finger delicately held the larva in place (Fig. 3). One proleg was amputated with a very sharp, blunt-pointed scissor, while the larva was held over the tube (Fig. 4). Between 0.10 and 0.14 ml. blood would flow from the cut and fall in the medium; pressure was not applied on the larval body, as the gut might rupture quite easily. After all larvae were bled, the total volume of blood collected was readily determined by a reading on the tube, graduated in 0.1-ml. units. From a dilution table available to the operator in the culture room the final amounts of the three components were read, and the proper additions made. The composition of the final culture was: 15% insect blood, 15% fetal bovine serum, and 70% maintenance medium.

After gentle mixing by pipette, the cell suspension was distributed in Petri dishes, each containing one Corning 22 × 22 mm. No. 1 coverglass. The volumes were 3.5 ml. for 60-mm. glass Petri dishes and 2 ml. for 35-mm. wettable polystyrene dishes (Falcon Plastics, Los Angeles). The cover glasses could be used for permanent preparations, after the culture was discarded. The Petri dishes were placed without delay in the incubator, in the gas mixture described in this paper. The medium was replaced every third day. We obtained good results without insect blood plasma, by using a replacement medium consisting of 30% fetal bovine serum and 70% medium 26C.

Culturing was performed with strictly aseptic techniques. The routine culture methods, the preparations of materials, etc., are not discussed here, since their description is readily available in most tissue culture textbooks.

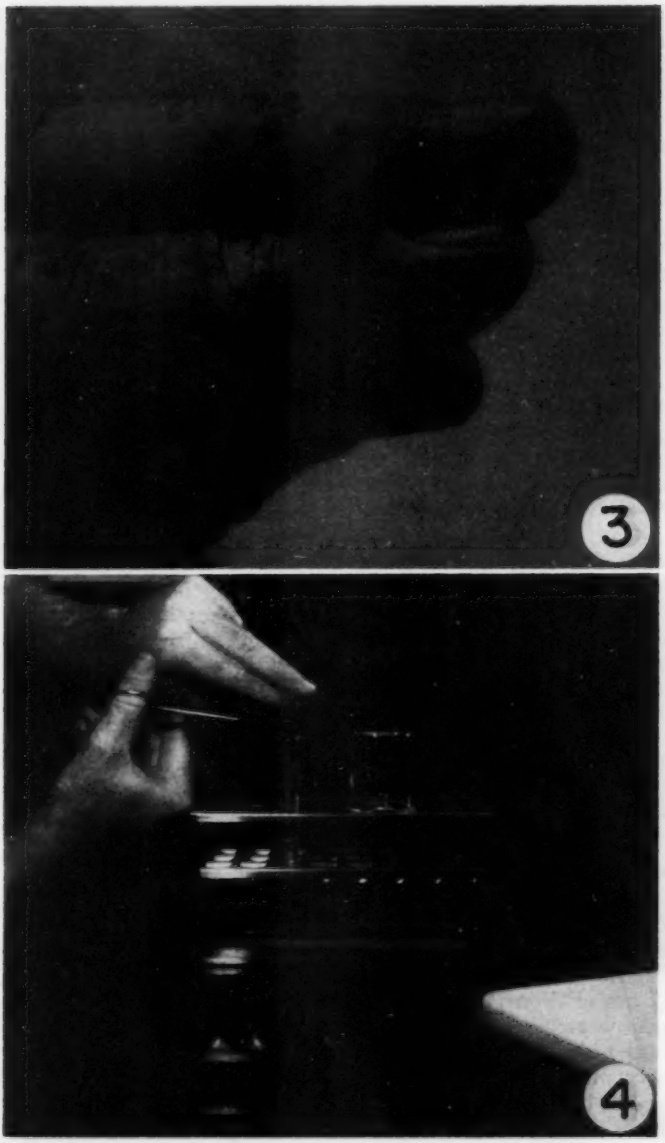


FIGURE 3. The handling technique for positioning of anesthetized larva of *Peridroma saucia* (Hübner). The prolegs are visible on the ventral surface of the larva.

FIGURE 4. The aseptic collection of larval blood (usually performed in a sterile culture room).

Results and Discussion

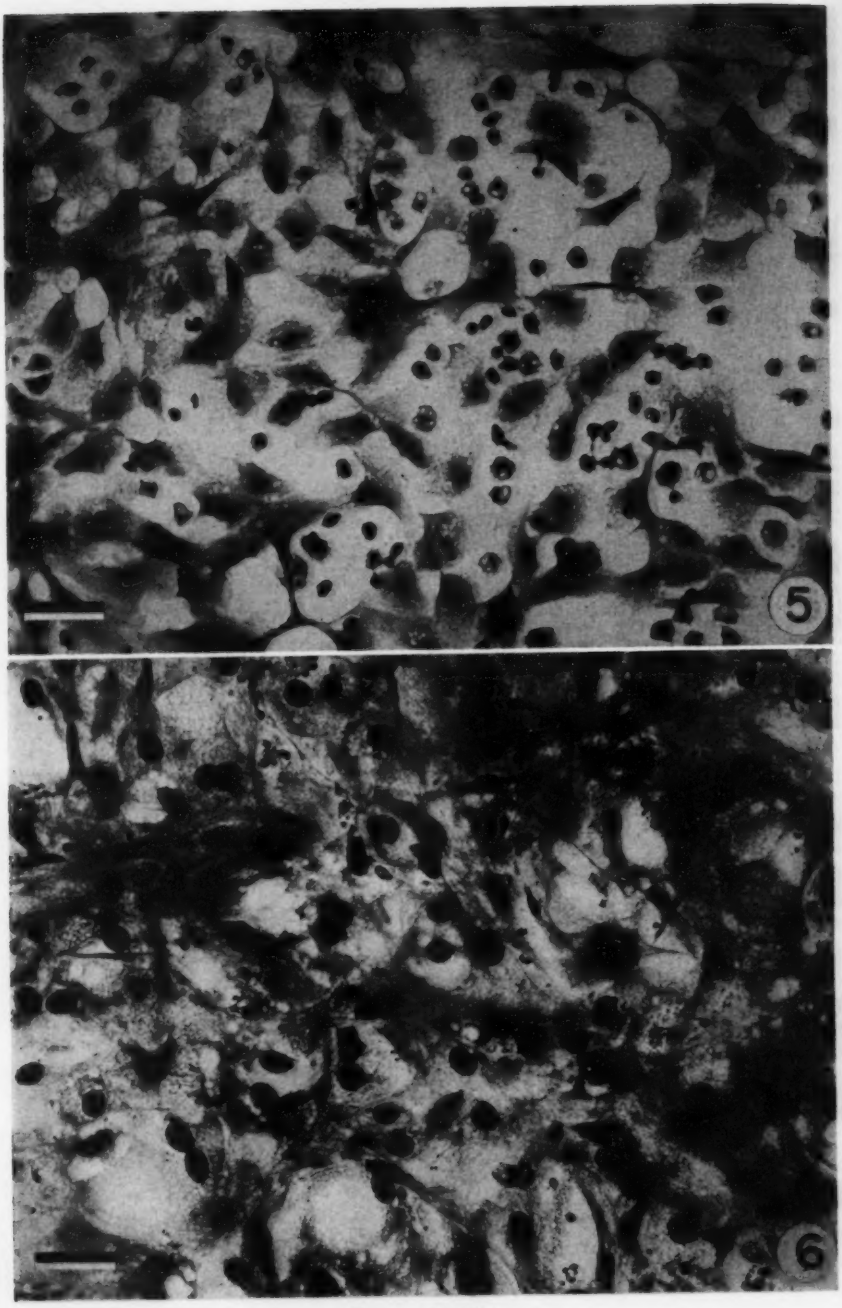
Cells cultured by the methods described here attached to glass readily and formed a sheet in less than 12 hours (Fig. 5). A number of cells remained floating in the culture vessel and did not settle on glass even after one to three days of incubation. These cells were eliminated when the medium was changed. The final cell sheet (Fig. 6) was composed mainly of plasmatocytes (amoebocytes of Wigglesworth, 1956), prohemocytes, and spheroidocytes (terminology according to Jones, 1959). Some degenerating cells were also present. These monolayers could be maintained in perfect condition for 10 to 15 days. Subcultures were not attempted.

Wigglesworth (1959; and in Campbell, ed., 1959) suggested that the cells migrating from the ovariole of Lepidoptera maintained *in vitro* are hemocytes formerly adherent to the tissues. The striking similarity of cells in cultures from the ovariole sheath of *Samia advena* Packard (Jones and Cunningham, 1960) with the hemocytes in our cultures seems to support this view: unfortunately, the two types of cultures have not been compared experimentally, to our knowledge. While occasional mitoses may be observed in cultures of insect hemocytes, proliferation is limited (or absent in some cases: Rosenberger and Jones, 1958), in the culture conditions available at present. Proliferation has been scanty also in cultures from the ovarian tissue of insect immatures (Grace, 1958; Jones and Cunningham, 1960), as compared with growth curves normally obtained in primary cultures of vertebrate tissue. Growth in insect tissue cultures is probably limited by the amount of endogenous ecdyson in tissue explants (growth "momentum": Schneiderman, in Campbell, ed., 1959).

In the cultures we have observed occasional variations that did not derive from changes in the cultural techniques, but were probably to be attributed to variability of the insect, as cell agglutination and violent tyrosinase activity. The larvae of *P. saucia* were fed with fresh alfalfa (*Medicago sativa* Linnaeus) and seasonal changes are known to occur in the plant. Furthermore, a certain variability in the insectary stock of *P. saucia* was inevitable. The presence of occasional aberrations is, of course, one of the main disadvantages of primary cell cultures.

Two criteria were used, in absence of considerable growth, to judge the suitability of a particular culture medium, substance, or method. One criterion was necessarily subjective: a judgment of the general appearance of the culture and of the conditions of the cell nucleus and cytoplasm. This appraisal was supplemented by a differential cell count, using a Howard disc inserted in one of the microscope eyepieces. A division of the cells into three categories was sufficient for our purposes: (I) prohemocytes; (II) plasmatocytes; and (III) spheroidocytes and other cells (including degenerating cells). Differential counts made on a sample of 62 cultures showed that the prevalent cell was the plasmatocyte, with a mean frequency of 80.35% of the cell population ($\sigma_{\bar{x}} = 1.015\%$) on the coverglass.

The plasmatocyte count was used in a series of tests (Table III) as a measure of the activity of a number of substances. This method may prove to be useful for bioassay *in vitro*. One should be reminded, of course, that at this point no direct extrapolation from cell to intact organism can be made without additional



FIGURES 5 and 6.

assay *in vivo*. Kinetin (6-furfurylaminopurine; Mann Research Laboratories, New York) was used at the level of 2 μg./ml. Increased rates of cell division after addition of low doses of kinetin to the culture medium have been reported for the epithelial outgrowth of human skin of the breast and for fibroblasts from a carcinoma of the breast (Orr and McSwain, 1957), and for *Paramecium caudatum* Ehrbg. (Guttman and Back, 1960). In our cultures, the addition of kinetin caused a decrease in plasmatocyte numbers. There was a proportional increase in cells of group III, and no change in the number of prohemocytes. An increase in mitotic activity was not noticed.

The addition of DL-carnitine hydrochloride (California Corporation for Biochemical Research, Los Angeles) at 5 μg./ml. did not cause appreciable changes in our cultures, while this vitamin was reported to increase the rate of mitosis in cultured chick-embryo fibroblasts (Fraenkel and Friedman, 1957).

TABLE III

Effect of some additives on the plasmatocyte ratio in vitro. The control cultures and the test cultures are from the same cell pool.

Test	Number of cultures	Per cent plasmatocytes		Level of significance (*t* test)
		$\bar{x}$	$\sigma_{\bar{x}}$	
Kinetin, 2 μg./ml.	25	80.34	1.39	8%
Controls	15	84.03	1.18	
Fly embryo extract, 10% EE_{50}	13	83.78	3.78	10%
Controls	4	67.48	11.66	
Carnitine, 5 μg./ml.	14	77.51	4.11	more than 10%
Controls	13	81.05	2.75	
Proteose-peptone 1 mg./ml.	18	81.62	1.25	more than 10%
Controls	9	82.93	1.61	

Proteose-peptone (Difco) at the level of 0.1% (Harris, 1959) did not alter significantly the ratios of the three cell groups when added to the cultures in presence of nondialyzed fetal bovine serum and homologous insect plasma. This suggests that peptides or other protein split products were present in sufficient amounts, in the serous fluids added to our medium. Tests with dialyzed serous fluids were not conducted, and this may be a promising avenue for future exploration.

The addition of an extract from house-fly embryos (*Musca domestica* Linnaeus) produced cultures with a high ratio of plasmatocytes. The extract was prepared from 8- to 10-hour-old eggs (containing almost fully developed embryos), homogenized in an equal volume of BSS, following a method similar to the one routinely

FIGURES 5 and 6. Hemocyte monolayers from *Peridroma saucia* (Hübner). The culture in Figure 5 is 12 hours old, the one in Figure 6 is 4 days old. The dominant cell in the monolayers is the plasmatocyte. Fixative: Duboscq-Brasil. Staining: Giemsa. Photography: V. G. Duran. Scale: 50 microns.

used for bovine embryo extract (EE_{50}). The test cultures received 10% of the Millipore-filtered extract. Unfortunately, in the course of this particular experiment an unexplained wide variation in plasmatocyte rates was observed, as evidenced by the large standard errors. However, there is some indication that the addition of fly embryonic extract had stimulative effects in our cultures.

The need for a medium poised at a low oxidation-reduction potential for the culture of insect hemocytes and probably other insect cells was clearly demonstrated by the analysis of differential cell counts made on two groups of cultures, one in which the medium showed melanin formation, another in which no melanin was formed. Melanin, which is known to act as an oxidation-reduction indicator (Figge, 1939), was formed in the medium supplemented with insect blood plasma, after exposure to oxygen. The results, summarized in Table IV, show that an increase in oxidation-reduction potential was connected with a decrease in plasmatocytes: a proportional increase was observed in cells of group III, in this case mostly degenerating cells. In addition, the cultures in which oxidation occurred were qualitatively inferior to the control cultures.

One more use of the hemocyte cultures should be mentioned briefly. Propagation of nuclear polyhedrosis virus is now possible in the plasmatocytes cultured

TABLE IV

The effect of a shift in oxidation-reduction potential on the plasmatocyte ratio in vitro

Group	Number of cultures	Per cent plasmatocytes		Level of significance (*t* test)
		$\bar{x}$	$\sigma_{\bar{x}}$	
Melanin formed	15	53.57	4.35	less than 0.1%
Controls	25	76.84	1.66	

as described here (Martignoni and Scallion, 1961). Inasmuch as the preparation of these cell cultures is relatively simple, we expect a number of studies on the interaction between virus and host at the cellular level, in the controlled conditions of the culture vessel.

The cooperation of Mr. James E. Milstead, who made numerous osmotic pressure determinations, and was most helpful in other phases of this work, and of Mr. Glenn L. Finney and Mrs. Mildred Schutter, who reared the five thousand larvae used for this study, is gratefully acknowledged. We also wish to thank Dr. Ralph I. Smith, who introduced us to the art of semi-micro osmotic pressure measurement.

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STUDIES ON THE HEXAPOD NERVOUS SYSTEM. IV. A CYTOLOGICAL AND CYTOCHEMICAL STUDY OF NEURONS AND THEIR INCLUSIONS IN THE BRAIN OF A COCKROACH, PERIPLANETA AMERICANA (L.)

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Inclusions commonly regarded as products of secretory activity have been described in the nerve cells of numerous and diverse insects (see reviews by Scharrer and Scharrer, 1954; Gabe, 1954; Van der Kloot, 1960). Much of this information has been obtained by employing selective, but poorly understood, staining reactions such as the aldehyde-fuchsin and chrome alum-hematoxylin techniques. Few attempts have been made to compare inclusions in different nerve cells of a single insect species using a variety of the more rigorous cytochemical staining procedures currently available. It might be expected that such a study would provide data germane to a problem which perennially faces the insect neurocytologist and neuroendocrinologist, namely, that of neuron classification. The present study is also considered basic to investigations of the fine structure of the cockroach nervous system now in progress at this laboratory.

Methods

By dissecting away the cranial and cervical integuments of live *Periplaneta* adults and late instar nymphs, the brain and retrocerebral complex were exposed. After severing the circumesophageal connectives, major nerves, and foregut distal to the corpora allata, the foregut was removed and carried to the appropriate fixative together with the brain and its associated retrocerebral complex. With practice this operation can be completed within two or three minutes, and certainly before any appreciable drying of the exposed organs occurs. A comparison with results obtained after exposing the brain and fixing it *in situ*, or after bathing the brain in Yeager's cockroach saline during the dissection did not justify modifying this procedure. Indeed, it was considered less damaging to remove the brain prior to fixation than after.

The choice of fixative was dictated by both the cytochemical staining technique intended, and the quality of cytological preservation required. Alcoholic and aqueous Bouin's, Lillie's acetic-alcohol-formalin (AAF; Lillie, 1954), 10% neutral formalin, and Helly's, were among those used. Preservation of neuronal inclusions in paraffin sections was best after Helly's. Following fixation, each specimen was dehydrated in half-hour changes of 70%, 95%, and absolute ethyl alcohol, cleared in methyl benzoate, washed briefly in benzene, and vacuum infiltrated with Fisher's Tissuemat (60–62° C.). Serial sections were cut at 5–7 μ. Adjacent sections were commonly mounted on alternate slides, thus allowing the cytological and cytochemical staining characteristics of the same inclusions to be compared.

Cytological features were elucidated by staining with Gomori's aldehyde-fuchsin (AF) as modified by Halmi (1952), or Bargmann's modification of Gomori's chrome alum-hematoxylin-phloxine (CAHP; Pearse, 1960). Additional information was obtained by staining Helly-fixed sections with the periodic acid-Schiff (PAS) method and counterstaining with sudan black B. While observing a particular region with a water-immersed objective (N.A. 1.25) several drops of acetone can be applied to remove the sudan black. This leaves the PAS-positive components. In this way the staining of inclusions by the two methods within a single cell was readily compared.

The cytochemical procedures used included the PAS reaction for polysaccharides after diastase digestion (Pearse, 1960), after aniline-acetic acid blocking (Lillie and Glenner, 1957), and after omitting periodic acid oxidation. In an attempt to decide whether lipids were responsible for PAS-positive reactions, freshly dissected brains were extracted with equal proportions of chloroform:methanol in a closed container at 60° C. for 24 hours. The PAS reaction was applied to gelatin-imbedded frozen sections of such extracted brains, as well as to frozen sections of brains which had been fixed in aqueous Bouin's first, and subsequently extracted with 60° C. pyridine. Frozen sections of aqueous Bouin-fixed brains served as controls.

Lipids were stained with sudan black B (SBB) in Helly-fixed paraffin sections, as well as in frozen sections of material which had been fixed either in aqueous Bouin's, or in neutral 10% formalin followed by postchromation (Baker, 1946). Controls consisted of aqueous Bouin-fixed, or unfixed brains, which had been extracted 24 hours either with 60° C. pyridine in a closed container, or with boiling chloroform:methanol in a reflux condenser. Baker's acid hematein (AH) test for phospholipids (Baker, 1946) was applied to gelatin-imbedded frozen sections. Helly-fixed paraffin sections were also stained with the copper phthalocyanin method (CPM) of Klüver and Barrera (Pearse, 1960).

RNA was localized in paraffin sections of brains and thoracic ganglia which had been fixed in Lillie's AAF for one hour, rapidly dehydrated in 95% and absolute ethyl alcohol, cleared in methyl benzoate, and vacuum-infiltrated. Constituents which stained with Einarson's gallocyanin-chromalum method for nucleic acids (pH 1.64; Pearse, 1960) after incubation for 1–2 hours in distilled water (37° C.), but which were not stained in adjacent sections incubated for the same period in 0.73% ribonuclease (recrystallized 5 times, without protease; Mann Laboratories), were considered RNA. These constituents are also present in neurons which have been fixed in alcoholic Bouin's, and they bind methylene blue at a pH of 2.5. Their strongly acidic nature is in accord with the hypothesis that they do, indeed, represent RNA.

To estimate the extent CAHP was dyeing the RNA component of neurons, paraffin sections of brains which had been fixed either in 80% ethyl alcohol or Helly's were stained after ribonuclease digestion. Sections which had been incubated in distilled water for identical periods were also stained with CAHP and served as controls.

The performic acid-alcian blue (PAAB) method of Adams and Sloper for disulfide groups (Pearse, 1960) was applied to paraffin sections of Helly-fixed material. Controls for the PAAB technique consisted of adjacent sections which

had been treated in the same manner as test sections, save for the omission of performic acid oxidation. The tetrazolium mixture of Barrnett and Seligman (1954) was used in the alkaline tetrazolium reaction (Pearse, 1960). This test was also applied to paraffin sections of Helly-fixed material.

Neurosecretory Material Produced by A-Cells

Of the many nerve cell bodies in the cockroach brain, only a relatively small number in the pars intercerebralis contains inclusions which stain both purple with AF and intensely blue-black with CAHP (Fig. 1A). Using the scheme of classification proposed by Nayar (1955), these would be called "A-cells." Due to the presence of stainable granules within the axoplasm, processes emanating from these cells can be traced to the first cardiac nerves, and thence to the corpora cardiaca (Scharrer, 1952; Arnold, 1960). Scharrer (1952) has demonstrated that this stainable material accumulates proximal to a transection of the first cardiac nerves, and is markedly depleted distally, suggesting that it is normally transported along the axons to the corpora cardiaca, which serve as neurohemal organs.

Different A-cells in the same individual are not invariably filled to the same extent with neurosecretory material (NSM). In the pars intercerebralis of some roaches, for example, A-cells which are packed with NSM were found adjacent to cells which contain only small, separate clusters of fine ($< 0.5\ \mu$ in diameter) neurosecretory granules. It is not improbable that this appearance is correlated with different phases of secretory activity.

Granules of NSM stain identically in the A-cells, in their processes within the brain and first cardiac nerve, and in the corpora cardiaca (Fig. 1B, C). A-cell NSM is poorly preserved by alcoholic fixatives. Unlike the situation in certain vertebrates (Sloper, 1955), loss of NSM is not appreciably prevented by post-fixing in 10% formalin after initial fixation in 80% ethyl alcohol prior to paraffin embedding. The loss of cockroach A-cell NSM appears to be due to the solvent action of alcohol, not to subsequent extraction by water. This is also indicated by the following data which show that NSM contains a lipid component.

Careful examination of adjacent Helly-fixed paraffin sections, one series stained with SBB, the other with CAHP, revealed that A-cell NSM is moderately sudanophilic. SBB can be removed readily with acetone, and the section can be restained, which further indicates that the sudanophilia is specific (Lillie, 1954). The sudanophilia of this NSM is also demonstrable in frozen sections of formal-bichromate or aqueous Bouin-fixed material. It is not apparent in frozen sections after hot chloroform:methanol extraction. A-cell NSM stains blue after Klüver and Barrera's CPM, and blue-black in frozen sections stained with Baker's AH (Fig. 1C). The latter reaction is abolished by hot pyridine extraction. The cytochemical evidence, therefore, suggests the presence of a phospholipid.

A-cell NSM is moderately PAS-positive in diastase-digested sections of Helly-fixed brain and retrocerebral complex. A positive reaction is also manifested by controls which have not been oxidized with periodic acid, but it is not as intense as that obtained after oxidation. After blocking exposed aldehyde groups with aniline acetate the PAS reaction is negative. The significance of the positive reaction is questionable. It could be caused by lipids (Pearse, 1960), or it could signify the presence of a carbohydrate moiety containing 1:2 glycol groups, which

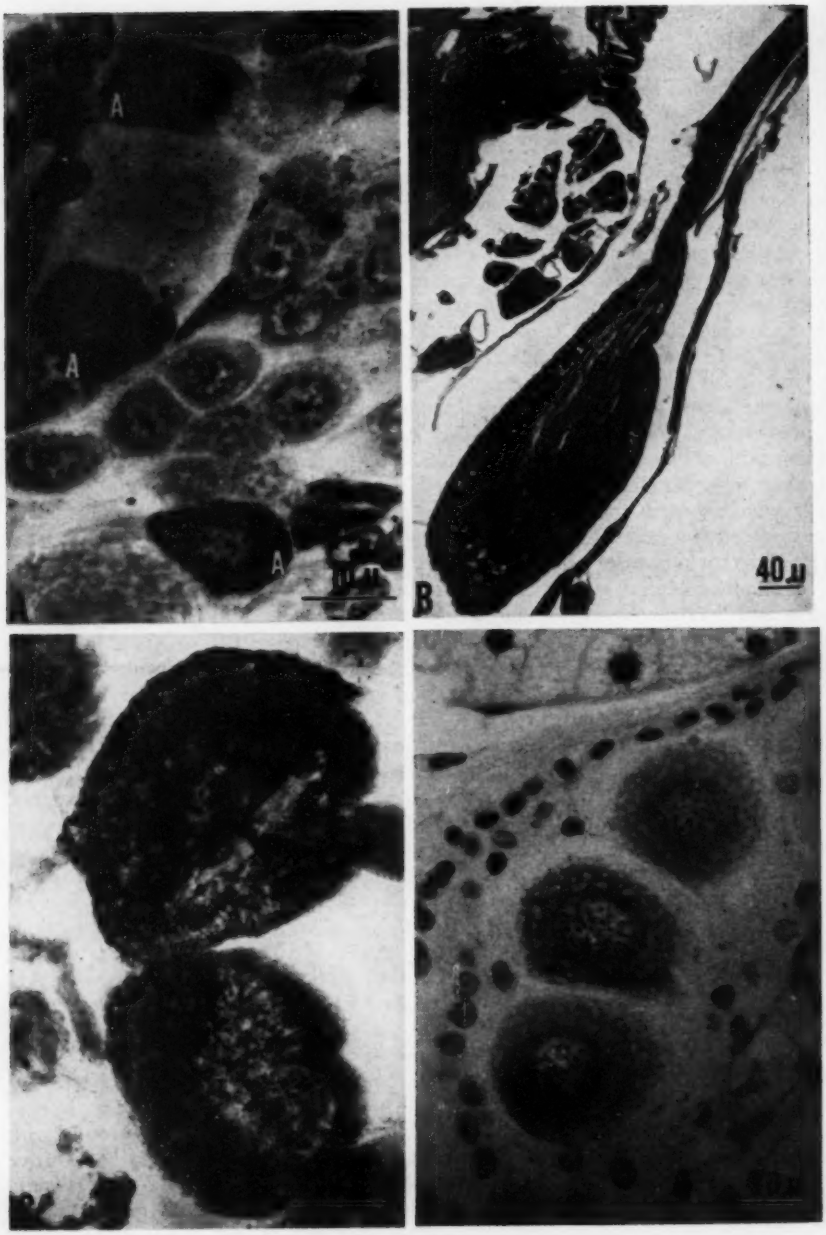

FIGURE 1.

occurs together with lipid. Since A-cell NSM is removed by lipid solvents, current cytochemical staining techniques do not permit a choice to be made between these possibilities.

A-cell NSM stains blue with the alkaline tetrazolium reaction, indicating the presence of protein reducing groups. Such an interpretation is further supported by the positive reaction noted with the PAAB method of Adams and Sloper for protein disulfide. The PAAB reaction is negative in controls which have not been oxidized with performic acid prior to staining with alcian blue. This is in agreement with the findings of Sloper (1957) in the cockroach *Leucophaea*. It should be emphasized, however, that this method stains other cytoplasmic inclusions in the nervous system besides A-cell NSM. Thus, cockroach gliosomes, first described by Scharrer (1939) and studied more recently by Pipa (1961), give a

TABLE I

Staining reactions of cytoplasmic inclusions in the brain of Periplaneta americana (L.)

Stain or test	A-cell NSM	Beta-granules	Delta-granules	RNA	Gliosomes
CAHP	Blue-black	Red	Pink or unstained	Blue-black	Blue-black
AF	Purple	Purple	Green	.....	Purple
Alkaline tetrazolium	Blue	Red	Gray	.....	Red
PAS (diastase)	++	++++	++++	.....	++++
PAS (unoxidized)	+	Faint, if at all	+	.....	+
PAS (blocked)	–	–	–	.....	–
SBB (frozen or Helly-fixed paraffin)	++	++++	++++	.....	Variable
SBB (lipid extracted)	–	–	–	.....	–
AH	++++	Faint, if at all	++++	.....	Variable
AH (hot pyridine extracted)	–	–	–	.....	–
Presence after alcohol fixation and paraffin method	Greatly reduced or absent	Greatly reduced or absent	Absent	Present	Greatly reduced or absent
CPM	++	++++	++++	.....	Variable
Gallocyanin-chromalum	–	–	–	++++	++++

positive reaction. Unlike neurosecretory granules, though, these inclusions stain red with the alkaline tetrazolium reaction. They also stain blue-black with Einarson's gallocyanin-chromalum, while A-cell NSM stains slightly, if at all (Table I).

RIBONUCLEIC ACID

A component cytochemically identifiable as RNA is present in the majority of neuronal perikarya. It is found distributed in patterns which vary in different

FIGURE 1. Photomicrographs of histological and cytological preparations; A, B, D fixed in Helly's; C fixed in 10% formalin followed by postchromation. (A) A-cells in pars intercerebralis stained with CAHP; (B) longitudinal section through corpus cardiacum and its nerve, stained with AF. Note intensely stained A-cell NSM in axons of nerve and in periphery of gland; (C) gelatin-imbedded frozen section of two corpora cardiaca; cross-section, stained with Baker's AH method for phospholipid; (D) three thoracic ganglion neurons stained with Einarson's gallocyanin-chromalum method for nucleic acids. A, A-cells.

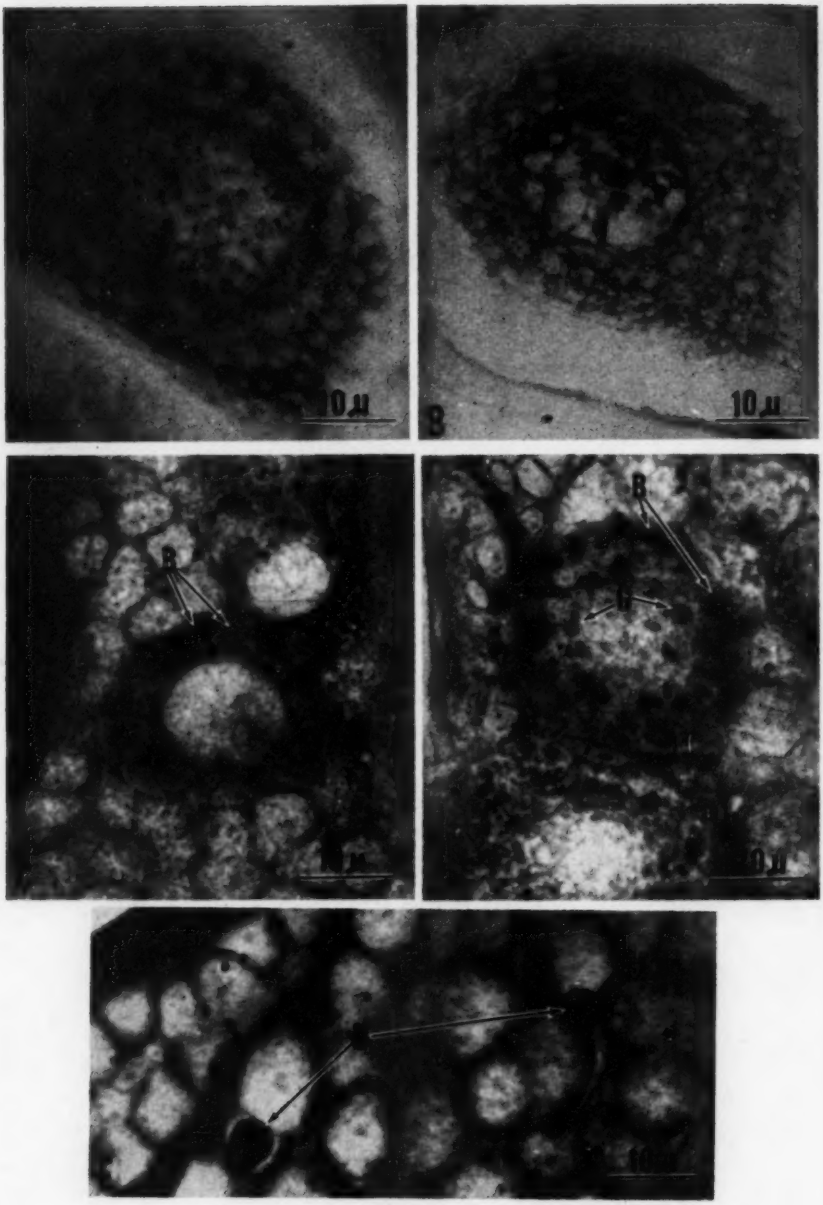

FIGURE 2.

neurons, commonly surrounding gallocyanin-chromalum-refractory spheroidal zones (Figs. 1D, 2A, B). Where these zones are reduced in diameter the distribution is more homogeneous. In agreement with the observations of Hess (1958), Arnold (1960), and Wigglesworth (1960), RNA was never found clumped to form well-defined Nissl bodies such as those described in vertebrate neurons. After alcohol fixation a vast majority of nerve cell perikarya in the brain stains blue-black with CAHP. That this staining is due to RNA is strongly suggested by the fact that it is abolished by ribonuclease digestion. Nerve cells of adjacent sections which were used as controls in this test stained after incubation in distilled water.

The perikarya of Helly-fixed neurons stain blue-black after CAHP, too. This is particularly noticeable when the phloxine counterstain is omitted. It is not surprising that this stainability remains essentially unaffected by ribonuclease, for this enzyme would hardly be expected to digest RNA in cells which have been fixed in a solution containing mercuric chloride. In all likelihood, though, the faint blue-black staining demonstrable in most Helly-fixed neurons after chrome alum-hematoxylin is also due to RNA. Fortunately, NSM within Helly-fixed A-cells stains more intensely than RNA after CAHP, and it is not difficult to distinguish these cells from other neurons found in the pars intercerebralis by this method.

Beta-Granules

A majority of the neurons contains inclusions which stain intensely with PAS, SBB, CPM, and AF, but which are not stained blue-black by CAHP (Fig. 2C, D). These inclusions range from 0.5 to 3.0 μ in diameter. They are especially well-preserved in frozen sections of formalin-bichromate-fixed material, and in Helly-fixed paraffin sections. They appear to vary in abundance in different neurons of the same brain, being most conspicuous in the axon hillock and periphery of each perikaryon, where they often occur in clusters. These inclusions were never found well-within the neuron process, and there was no evidence to support the contention that they represent neurosecretory products. Failure to find cytological evidence of axonal transport, however, does not eliminate the possibility that these inclusions represent a visible manifestation of products which leave the perikaryon via a different route, or in an invisible form. Although not all of these inclusions may be of the same type, they shall be designated beta-granules for the sake of discussion.

When counterstained sufficiently and not destained excessively, beta-granules demonstrate a slight phloxinophilia after CAHP, but this is less intense than that seen in nucleoli present in the same cell. They also stain red after the alkaline tetrazolium reaction, and are PAAB-negative.

The larger of these inclusions stained slightly, if at all, during the Baker AH procedure, whereas NSM of the A-cells, and inclusions believed to be mito-

Figure 2. Photomicrographs of cytological preparations; A, C, D, E fixed in Helly's; B in Lillie's AAF. (A) thoracic ganglion neuron stained with Einarson's gallocyanin-chromalum method for nucleic acids. Note distribution of RNA around unstained spheroids which are believed to represent loci of Golgi apparatus; (B) similar to (A), but after alcoholic fixation; (C) Beta-granules stained with PAS and SBB; (D) Golgi apparatus and beta-granules in same neuron perikaryon, stained with PAS and SBB; (E) Delta-granules stained with PAS and SBB. B, beta-granules; D, delta-granules; G, Golgi apparatus.

chondria, were intensely stained in the same sections. Beta-granules stain with SBB and PAS in gelatin-imbedded frozen sections after formalin-bichromate or aqueous Bouin fixation. They are seldom found in frozen sections of brains which have been both fixed and extracted with hot chloroform:methanol. The larger of these inclusions are still abundant and stainable with SBB and PAS in frozen sections of material which has been fixed in aqueous Bouin's and subsequently extracted with hot chloroform:methanol, or hot pyridine. These data suggest that beta-granules contain a lipid or glycolipid which is either not predominantly phospholipid, or from which phospholipid has been removed during fixation. They are rendered relatively resistant to the action of lipid solvents by formalin fixation, indicating chemical association with a protein.

Although beta-granules stain alike, it would be gratuitous to assume that they are chemically identical. The cytochemical tests used are generally considered specific for certain classes of compounds if appropriate controls are employed, but they do not identify compounds within each class. The presence of a common chemical group within two inclusions can hardly be considered proof that the two are chemically identical.

It might be argued that certain, if not all of these "beta-granules" represent mitochondria. Though this has not been dismissed as a possibility, it seems unlikely in view of the fact that inclusions believed to be mitochondria on the basis of electron microscope data generally stained yellowish green rather than purple with AF. Mitochondria also stain less intensely with PAS than do beta-granules. After Baker's AH test mitochondria stain blue-black, whereas the larger beta-granules within the same cells stain slightly, if at all. Preliminary electron microscope data which are currently at hand likewise indicate that beta-granules differ in fine structure from "typical" mitochondria found within the neuron.

Golgi Complex

When formalin-bichromate-fixed frozen sections, or Helly-fixed paraffin sections of neurons are stained with SBB, not only are the homogeneously-stained beta-granules mentioned above made evident, but sudanophilic crescent-shaped bodies and "hollow" spheroids frequently appear in the same cells. Each of these is comprised of an "externum" which stains intensely with SBB, and an "internum" which is variably sudanophilic (Figs. 2D, 3A). The "externa" are especially well-revealed in paraffin sections stained with Klüver and Barrera's CPM (Fig. 3B).

When Helly-fixed paraffin sections that have been stained with PAS and counterstained with SBB are flooded with acetone, SBB is removed, and the following observations can be made during the destaining process: (1) The beta-granules described above are both intensely PAS-positive and sudanophilic. Thus, when SBB has been removed they are still clearly evident. (2) The "hollow" spheroids and crescent-shaped structures which stained with SBB become indiscernible. Unlike beta-granules these structures stain slightly if at all with PAS. (3) Occasional beta-granules occur within the "hollow" spheroids or crescents. When SBB has been removed, red-staining inclusions of variable size can be seen within a zone which is either PAS-negative, or only very slightly PAS-positive.

The Golgi apparatus in the neurons, then, consists of sudanophilic, crescent-

shaped or spheroidal "externa" which are probably manifestations of lamellar membrane systems (Beams *et al.*, 1953; Palay and Palade, 1955; Bern *et al.*, 1961). These are frequently closely associated with variably sudanophilic "interna" which may contain beta-granules. The beta-granules are sudanophilic, intensely PAS-positive, and stain purple with aldehyde fuchsin. A summary of their staining characteristics is given in Table I.

Nerve cells which have been stained by techniques used to demonstrate RNA (*i.e.*, gallocyanin, cresyl violet) commonly present a variably "mottled" appearance (Figs. 1D, 2A, B). As previously noted, this is due to the staining of RNA

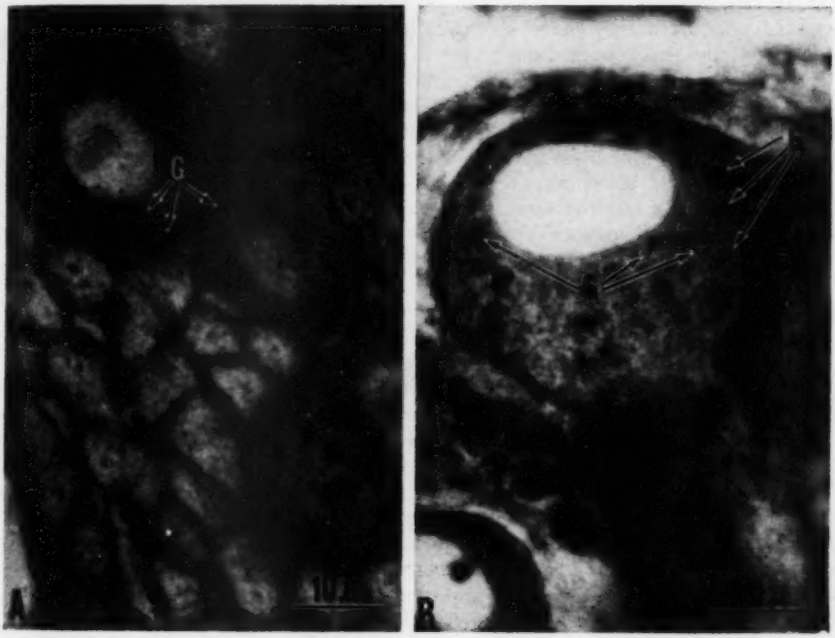

FIGURE 3. Photomicrographs of cytological preparations; fixed in Helly's. (A) Golgi apparatus stained with SBB. (B) Neuron stained with CPM; Golgi "externa" evident in cytoplasm. G, Golgi bodies.

which surrounds unstained spheroidal zones. An examination of adjacent Helly-fixed paraffin sections of the same neurons, one series stained with chrome alum-hematoxylin, the other with SBB, led to the conclusion that these spheroidal zones represent the loci of the Golgi apparatus which are devoid of ribosomes (*i.e.*, are present as "agranular reticula"; Palay and Palade, 1955; Palay, 1958; Bern *et al.*, 1961).

When frozen sections of aqueous Bouin-fixed, pyridine-extracted brains are stained with SBB and examined, the Golgi apparatus is no longer demonstrable. The cytoplasm now appears finely granular, save for the occurrence of the larger

beta-granules which continue to stain with SBB and which will also stain intensely with PAS. This may be taken as additional evidence that the Golgi apparatus and larger beta-granules are not one and the same structure.

Delta-Granules

Within the perikarya of certain of the smaller neurons (nuclear diameters of 10 to 15 μ) single, conspicuous, spherical inclusions are consistently encountered (Fig. 2E). These inclusions, here termed delta-granules, range from 2 to 5 μ in diameter. They occur within the brains of late instar nymphs, as well as in adult males and females. It is uncertain whether they are limited in occurrence to special cells, or if they occur sporadically in different neurons. Cells containing delta-granules may occur singly or in small groups. They were seen most frequently in the pars lateralis, less commonly in the pars intercerebralis.

Delta-granules do not stain homogeneously, for areas which fail to stain, or which stain unlike the remainder are noticeable at their centers. Delta-granules may stain faintly pink after CAHP, or they may fail to stain altogether. They are not fuchsinophil, but stain green with the counterstain employed in the AF technique. Their failure to stain purple, and the fact that they stain blue-black after Baker's AH distinguishes them from beta-granules (Table I). Since delta-granules no longer stain with AH after pyridine extraction it is likely that they contain appreciable phospholipid.

Discussion

The inherent limitations of staining reactions as criteria for categorizing cytoplasmic inclusions are painfully apparent. The fact that two structures stain alike does not necessarily mean they are chemically identical. When more rigorous cytochemical staining techniques are employed one may be led to conclude that two inclusions contain the same class of compounds, but it is seldom possible to tell precisely what the compounds are. It is common knowledge that structures which play disparate roles in the economy of the cell may contain identical classes of compounds, and may therefore stain identically. When two inclusions fail to stain alike, however, it is logical to consider them chemically or physically dissimilar.

In light of these precepts the advisability of using a diversity of staining techniques becomes obvious. In the present case such an approach has led to the recognition of three types of neuronal inclusions which are believed to exist in addition to mitochondria, ribosomes, and the agranular reticulum (*i.e.*, Golgi apparatus). To facilitate discussion these are called A-cell NSM, beta-granules, and delta-granules.

A-cell NSM was found in certain neurons of the pars intercerebralis, as well as in the corpora cardiaca. This is in agreement with the observations of Scharrer (1952) and Arnold (1960). The histochemical evidence indicates that this NSM contains a lipoprotein. This has also been noted in certain Lepidoptera (Rehm, 1955) and Hemiptera (Nayar, 1955). The fact that A-cell NSM stains intensely with Baker's AH before pyridine extraction, but not after, suggests the presence of phospholipid. The moderately PAS-positive reaction which this NSM exhibits is of unknown significance. That it is not due to glycogen is certain, but it

could be caused by a lipid component, or by a carbohydrate moiety associated with lipid. Since lipid solvents will remove A-cell NSM, current cytochemical staining procedures do not permit a choice to be made from among these possibilities.

As indicated by Sloper (1957) NSM produced by insect A-cells resembles that produced by certain neurons of the vertebrate hypothalamus, inasmuch as it shows evidence of containing a protein or polypeptide rich in cystine or cysteine. In other respects it differs significantly from vertebrate NSM, as reported by Sloper (1955), and Howe and Pearse (1956). These workers concluded that vertebrate NSM is mainly protein or polypeptide in nature, there being no indication of lipid. Their findings are in opposition to those of Schiebler (1952) who interpreted his histochemical data as indicating a glycolipoprotein complex.

Beta-granules are of variable size. Although they stain alike, it is possible that future study may reveal this to be a heterogeneous category. They occur in variable abundance in the majority of neuron perikarya, and are most conspicuous in the periphery, especially in the axon hillock region. A resumé of their staining characteristics is given in Table I. Beta-granules differ from mitochondria by staining intensely PAS-positive, and by staining purple with AF. More significantly, preliminary electron microscope data indicate that they differ from mitochondria in fine structure. Beta-granules were never found well-within the neuron process. If they do represent a type of neurosecretory material, they must be altered in size or stainability before passing out of the perikarya. Perhaps they are comparable to the chromophilic intraneuronal granules described by Thomas (1951).

Although it is not the intention of this study to enter into the "Golgi controversy" (Baker, 1957; Lacy and Challice, 1957; Palay, 1958), a comparison between locust "lipochondria" and structures noted here would seem appropriate. According to the interpretation of Shafiq (1953, 1954) and Shafiq and Casselman (1954) the classically described Golgi apparatus is an artifact resulting from the deposition of osmium or silver on the surfaces of homogeneous spheroids or "lipochondria" during impregnation. Malhotra (1956), however, reported seeing the binary spheroidal Golgi apparatus not only in osmium or silver impregnations, but also in living insect neurons, as well as in cytological preparations stained with SBB. He did not describe neuronal perikaryal inclusions other than mitochondria, Golgi binary spheroids (=lipochondria), and RNA particles.

The present account concurs with Malhotra's finding that in cytological preparations stained with SBB the Golgi apparatus is demonstrable as intensely stained crescentic "externa" associated with less intensely stained "interna." This appearance, then, is not entirely an artifact due to the deposition of osmium or silver on otherwise homogeneous spheroids. Electron microscope investigations have also revealed variably crescentic lamellar membrane systems ("agranular reticula") as part of the neuron Golgi apparatus (Beams *et al.*, 1953; Palay and Palade, 1955; Palay, 1958; Bern *et al.*, 1961), thus providing a probable fine-structure basis for the Golgi "externum" visible with the light microscope. The fact that no RNA could be demonstrated within the spheroidal zones ("externum" plus "internum") in the present work supports the contention that they may, indeed, represent the loci of the agranular (ribosome-free) reticula.

It is not clear whether by the term "lipochondria" Shafiq meant the spheroidal

zones (Figs. 1D, 2A, B) which represent the loci of the Golgi apparatus ("externum" plus "internum"), the larger beta-granules noted here, or both. It is quite certain, however, that the Golgi apparatus and the beta-granules are not one and the same structure. This interpretation is also supported by preliminary electron microscope data currently at hand.

The functional significance of the conspicuous delta-granules is likewise unknown. They were found solely in the perikarya of smaller neurons; never in the processes. A comparison between the staining affinities of these and certain other inclusions within the neurons is made in Table I.

The cytomorphology of neurons varies in different cells of the same brain. It also varies in the same group of cells in different brains. This variability is expressed as differences in the degree of Golgi apparatus elaboration, RNA distribution, and in number and size of inclusion granules. If we are willing to accept these static appearances as evidence for secretory activity, then it would seem that a majority of the neurons in the brain are secreting. Such cytomorphological features need not necessarily be correlated with neurosecretion, however, for they could conceivably reflect axoplasm replacement (Weiss and Hiscoe, 1948), or changes associated with ageing (*i.e.*, accumulation of lipofuscin).

On the basis of the cytological data presented here it would be premature to conclude that beta- and delta-granules represent neurosecretory products. It is also entirely possible that secretion may occur without the production of visible granules. Clearly, reciprocal physiological information is essential before this matter can be decided.

It is a pleasure to express my gratitude to Professor Berta Scharrer, Albert Einstein College of Medicine, New York, and to Professor Roderick Craig, University of California, Berkeley, for critically reading the manuscript, and for numerous suggestions. This does not imply endorsement or responsibility on their part for any conclusions or views presented.

Summary

1. A variety of cytological and cytochemical staining reactions has been applied in a study of neuronal inclusions in the brain of the American cockroach. Three types of cytoplasmic inclusions, which exist in addition to mitochondria, ribosomes, and the Golgi apparatus, are characterized. For discussion purposes these are called A-cell NSM, beta-, and delta-granules.

2. The cytochemical staining reactions used indicate that A-cell NSM is a cystine- or cysteine-rich phospholipoprotein.

3. Perikaryal inclusions which stain intensely with PAS, SBB, CPM, and AF, but which are not stained blue-black with CAHP, occur in the majority of neurons. These are called beta-granules. Despite similarities in stainability, there is a possibility that these inclusions are not all of one type. They clearly differ from the Golgi apparatus, and it is also highly unlikely that they are "typical" mitochondria. The cytochemical data suggest that beta-granules contain a lipid or glycolipid which is either not predominantly phospholipid, or from which phospholipid has been removed during fixation. The fact that they are rendered relatively

resistant to lipid solvents by formalin fixation indicates chemical association with a protein.

4. Delta-granules occur as single, conspicuous inclusions within the perikarya of certain of the smaller neurons. Unlike beta-granules, they stain green with AF. They appear to consist of a lipoprotein or glycolipoprotein which contains phospholipid.

5. The Golgi apparatus consists of sudanophilic crescent-shaped or spheroidal "externa" frequently closely associated with variably sudanophilic "interna" which may contain beta-granules. The "mottled" appearance of nerve cells which have been stained to demonstrate RNA appears to be due to the unstained Golgi spheroids.

6. The limitations of staining reactions as criteria for categorizing cytoplasmic inclusions, and the significance of cytomorphological features as indices of neurosecretory activity are discussed.

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UREASE ACTIVITY IN TRYPANORHYNCH CESTODES[1]

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The hydrolysis of urea has been reported as an activity of tissue preparations from a number of invertebrate animals. Urease activities were detected in a lamellibranch (*Mytilus edulis*), a gastropod (*Helix pomatia*), an oligochaete (*"Lumbricus agricola"*), and a decapod crustacean (*Astacus fluviatilis*) by Przylecki (1922); in five species of marine molluscs by Albrecht (1921); in the horseshoe crab (*Limulus polyphemus*) by Loeb and Bodansky (1926, 1927); in the eggs and developing embryos of a sea urchin (*Strongylocentrotus purpuratus*) by Brookbank and Whiteley (1954); in the larvae and larval secretions of a blowfly (*Lucilia sericata*) by Robinson and Baker (1939); in a sipunculid (*Sipunculus nudus*) by Florkin and Duchateau (1942). The latter authors also reported activities in tissues of a crayfish (*"Ecrevisse"*) and a lobster (*"Homard"*) (Florkin and Duchateau, 1943). Brunel (1938) also reported urease activity from *Mytilus edulis*, while Baldwin and Needham (1934) detected activity in the nephridium of *Helix pomatia*.

Several species of parasitic nematodes have been reported to possess a urease. Rogers (1952) demonstrated urease activity in *Nematodirus* spp., and stated that lesser activities were found in *Ascaridia galli* and *Haemonchus contortus*. Savel (1955) studied urease in several tissues of *Ascaris lumbricoides*.

Apparently there have been no previous observations of urease activity in flatworms. Van Grembergen and Pennoit-deCooman (1944) failed to detect activity in a trematode (*Fasciola hepatica*) and two cestode parasites of mammals (*Moniezia benedeni* and *Taenia pisiformis*).

The present paper will show that certain trypanorhynch tapeworms from elasmobranch hosts possess urease of quite remarkable levels of activity.

Materials and Methods

Infected elasmobranches were obtained through the courtesy of the Supply Department of the Marine Biological Laboratory. All the fishes came from a trap in Buzzard's Bay, Massachusetts, and were identified by reference to the paper of Bigelow and Schroeder (1953).

Fishes were killed by blows on the head, and cestodes were immediately collected from the spiral valves. A saline solution composed of 250 m*M* NaCl, 4.4 m*M* KCl, 5.1 m*M* $CaCl_2$, and 2.9 m*M* $MgCl_2$ was used for rinsing and incubating the worms. Following several saline rinses to remove adhering materials, the worms

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were incubated for 2–5 hours at 20° C. Such treatment was found to render the tetraphyllid cestode, *Calliobothrium verticillatum*, free of detectable endogenous urea (Read *et al.*, 1959). Subsequent to this incubation period, worms were blotted on hard filter paper and the desired amount of tissue weighed on a torsion balance.

Homogenates of worm tissue were prepared in TenBroeck tissue grinders of suitable size immersed in a cracked-ice bath. The homogenates were 1:9 (w/v) dilutions of wet tissue, the diluent being an ice-cold solution of 5 m*M* disodium ethylenediamine tetraäcetate (EDTA).

Urease activity of tissue homogenates was assayed by a modification of the method described by Sumner (1955), which measures the ammonia produced from the hydrolysis of urea. A complete reaction mixture consisted of 200 microliters of a urea solution of appropriate concentration, 200 microliters of a 100 m*M* buffer

TABLE I

Urease activities in cestodes of elasmobranchs

Worm species	Host	No. determinations	Tissue activity (mean ±s.e.)
Tetraphyllidea			
Calliobothrium verticillatum	*Mustelus canis*	8	ND
Phyllobothrium lactuca	*Mustelus canis*	4	ND
Phyllobothrium foliatum	*Dasyatis centrura*	4	ND
Onchobothrium pseudo-uncinatum	*Dasyatis centrura*	4	ND
Inermiphyllidium pulvinatum	*Dasyatis centrura*	7	ND
Orygmatobothrium dohrnii	*Carcharias taurus*	4	ND
Disculiceps pileatum	*Carcharinus obscurus*	4	ND
Trypanorhyncha			
Lacistorhynchus tenuis	*Mustelus canis*	8	3,330± 40
Grillotia erinaceus	*Raja ocellata*	7	ND
Pterobothrium lintoni	*Dasyatis centrura*	8	33,290±610

Five-minute incubations at 20° C.
ND: not detected, less than 25 micromoles urea hydrolyzed per gram wet tissue per hour.

solution (phosphate buffer, pH 7.0, unless otherwise indicated), and 100 microliters of 10% tissue homogenate. The reaction was initiated by blowing in the homogenate and stopped by blowing in 200 microliters of 20% trichloroacetic acid (TCA). Control preparations for the assay included (1) untreated homogenate in a reaction mixture lacking urea, (2) heat-inactivated homogenate incubated in the presence of urea, (3) zero-time preparations with TCA added prior to the addition of homogenate, and (4) diluent alone (no tissue) in the presence of urea. The latter preparation never yielded detectable ammonia; the other control preparations gave low (about 25 micrograms of ammonia nitrogen per gram wet tissue), and practically identical, ammonia values. All assay values are reported as corrected for the maximum tissue blank value.

Ammonia produced in the reaction mixtures was liberated by the addition of 1.5 milliliters of a saturated solution of potassium carbonate and captured in 1 N

H_2SO_4 by the microdiffusion method of Seligson and Seligson (1951). Ammonia was determined by the procedure of Lang (1958).

Protein was estimated by a modification of the Lowry method (Lowry *et al.*, 1951) using crystalline bovine serum albumin (Armour and Company) for comparison.

Results are reported as tissue activities or as specific activities, here defined as micromoles urea hydrolyzed per gm. wet tissue weight per hour and micromoles urea hydrolyzed per mgm. protein per hour, respectively, assuming that two micromoles of ammonia recovered are equivalent to one micromole of urea hydrolyzed.

Additional experimental procedures will be described in context.

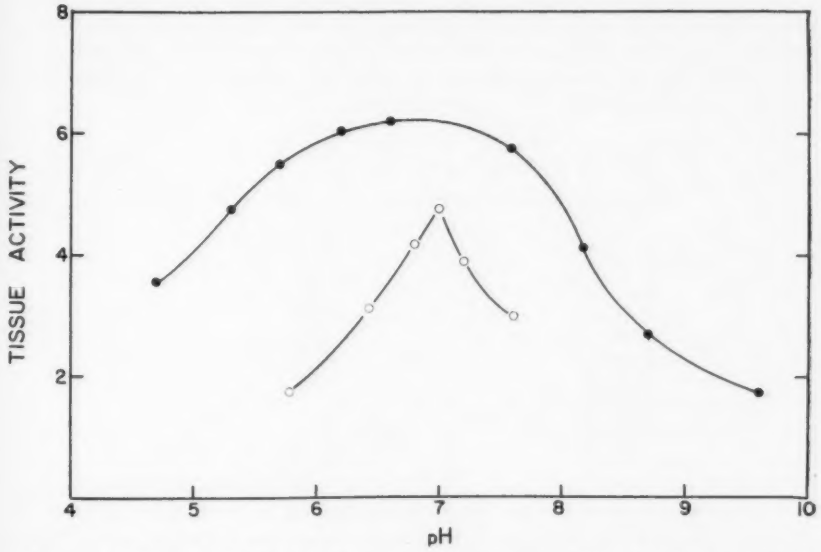

FIGURE 1. Effect of pH on *Lacistorhynchus* urease activity. Tissue activity as micromoles urea hydrolyzed per gram wet tissue per hour in thousands. Open circles with phosphate buffer; closed circles with Tris-maleate. One-minute incubations at 20° C. Each point represents the mean value of four determinations.

RESULTS

A. *Distribution of urease activity among cestodes of elasmobranch fishes*

Read *et al.* (1959) have shown that urea is an important osmotic constituent of certain Tetraphyllidea, a tapeworm group parasitizing elasmobranch fishes; data derived from studies with [^{14}C] urea indicate that in tetraphyllid cestodes urea is a metabolically inactive compound (Simmons *et al.*, 1960).

Ten species of tapeworm from elasmobranches were examined for urease activity by the procedures just described; results of the assays are given in Table I. Of seven tetraphyllids examined, none exhibited activity, while two of three trypanorhynch species examined were quite active in hyrolyzing urea.

Additional *Pterobothrium* material was not forthcoming, and further studies with tissue preparations were made with the more readily available *Lacistorhynchus*.

B. *Studies of Lacistorhynchus urease in tissue homogenates*

Effect of pH on activity

Figure 1 shows activity curves obtained when Sørensen's phospate buffer and tris(dimethyl)aminomethane (Tris)-maleic acid buffer (Gomori, 1955) are employed in the assay.

The very sharp optimum observed at pH 7.0 in phosphate buffer is characteristic also of jackbean urease (Sumner, 1951). Apparently there have been no studies with jackbean urease employing Tris-maleate although Wall and Laidler (1953) found that crystalline jackbean urease showed a marked optimum at pH 8.00 in Tris-sulfuric acid buffer. *Lacistorhynchus* homogenates show no such optimum in Tris-sulfuric acid when the pH is varied between 7.4 and 9.0 (Table II).

TABLE II

Urease activity of Lacistorhynchus homogenates with Tris-H_2SO_4 Buffer

pH	Tissue activity (mean ±s.e.)
7.4	3370 ± 40
7.8	3160 ± 50
8.0	2690 ± 42
8.2	2120 ± 50
8.6	2080 ± 43
9.0	1920 ± 20

One-minute incubations at 20° C.
Activities are mean values of four determinations.

Although the worm activities in Tris-maleate are considerably elevated above those obtained using phosphate buffer, especially on either side of pH 7.0, the latter buffer was employed in subsequent experiments since most studies of urease activities from other sources have employed phosphate buffer.

Activity as a function of enzyme concentration

Linearity of activity as a function of protein concentration was observed over the range examined, 0.068–1.09 mgm. protein (Fig. 2).

Effect of substrate concentration

Table III gives activities of *Lacistorhynchus* homogenates with increasing urea concentrations. The saturating concentration range, observed from about 250 m*M* to 350 m*M* is quite interesting in view of the fact that the urea concentration in the host blood is about 330 m*M* and that of the gut contents is similar (Read *et al.*, 1959).

The worm enzyme may be less susceptible to product inhibition than jackbean enzyme, since the latter exhibits decreasing activities in urea concentrations about

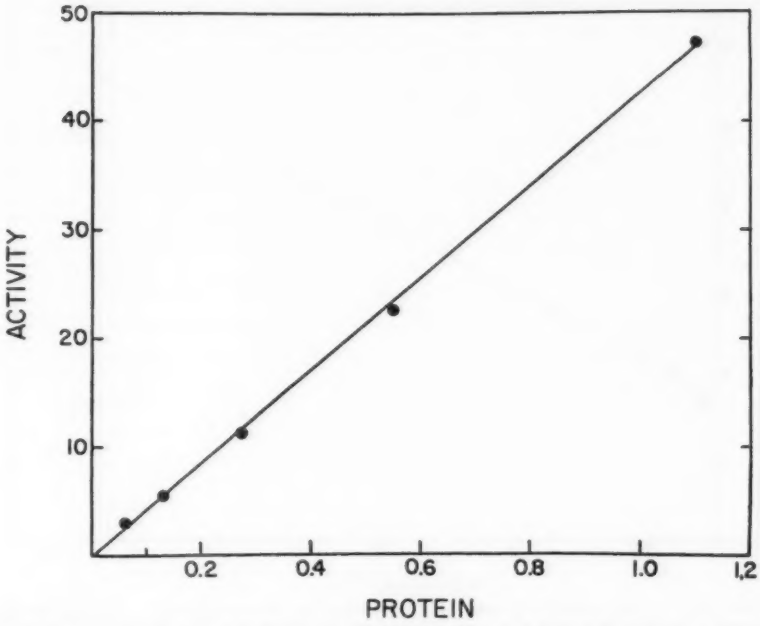

FIGURE 2. Urease activity of *Lacistorhynchus* as a function of protein concentration. One-minute incubations at 20° C. Activity as micromoles urea hydrolyzed per hour. Protein in milligrams. Each point represents the average of duplicate determinations.

250 m*M*–330 m*M*, presumably due to inhibition by the ammonium ions produced (Wall and Laidler, 1953).

Data obtained with the tapeworm urease give good agreement with Michaelis-Menten kinetics when plotted by the method of Lineweaver and Burk (1934), over

TABLE III

Urease activity of Lacistorhynchus homogenates with varying urea concentrations

Urea concentration m*M*	Tissue activity
6.25	835
12.5	1300
25.0	1780
50.0	2140
100.0	2490
200.0	2783
250.0	3108
300.0	3250
350.0	3150
400.0	2967

One-minute incubations at 20° C.
Activities are the average of duplicate determinations.

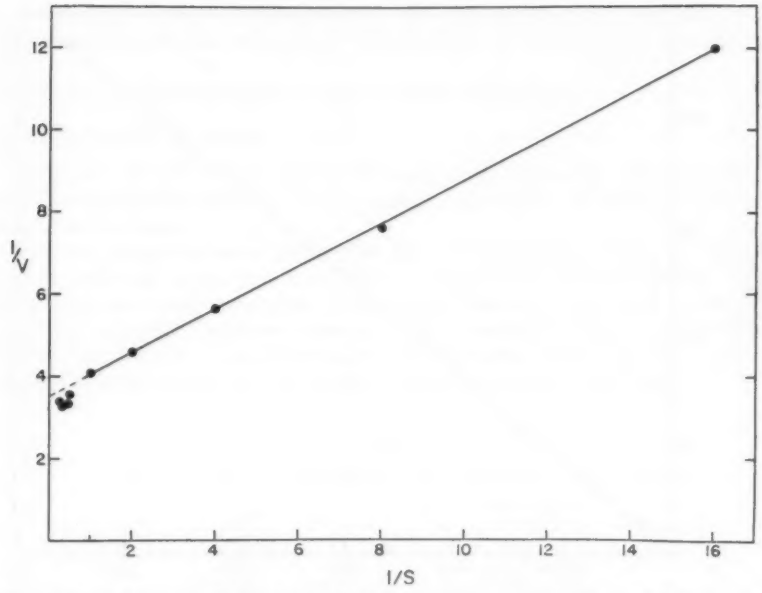

FIGURE 3. Lineweaver-Burk plot of *Lacistorhynchus* urease activity. Velocity reciprocal as (micromoles urea hydrolyzed per gram wet tissue per hour)$^{-1} \times 10^4$. Substrate reciprocal as (millimolar urea)$^{-1} \times 10^2$. One-minute incubations at 20° C. Each point represents the average of duplicate determinations.

the lower substrate concentration range (6.25 m*M* to 100 m*M* urea) (Fig. 3). From 200 m*M* to 400 m*M*, the velocity observed is in excess of that expected.

Activity in saturating substrate concentration

When incubated in the presence of 300 m*M* urea, *Lacistorhynchus* urease activity follows zero-order kinetics for at least four minutes (Fig. 4).

Enzyme stability

When held at 0° C. in the saline solution previously described, *Lacistorhynchus* remain viable for at least 110 hours, the total period of observation. Homogenates freshly prepared from such worms retain full activity (Table IV).

Homogenate preparations *per se* are, however, quite labile; about 93% of the activity is lost in 24 hours at 20° C., and the rate of decay is only little different at 0° C. (Table IV). The loss of specific activity with a concomitant loss of tissue activity is highly suggestive that the decreased activity is due to inactivation and not to general proteolysis.

Storing of whole worms at −20° C. followed by thawing at 0° C. resulted in a loss of activity in homogenates prepared from such material of about 90%; freezing, followed by lyophilization resulted in complete loss of activity.

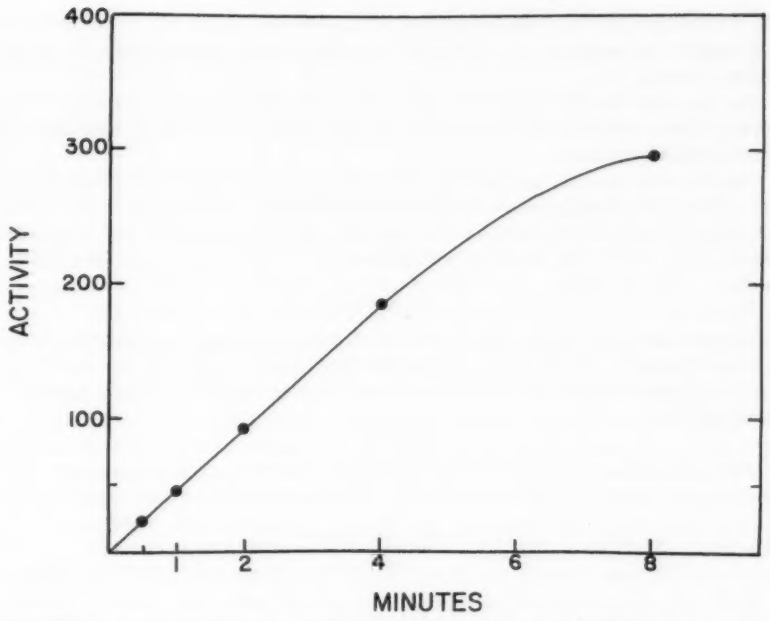

FIGURE 4. Activity of *Lacistorhynchus* urease with time. Incubations at 20° C. Urea concentration 300 m*M*. Activity as micromoles urea hydrolyzed per milligram protein. Each point represents the average of duplicate determinations.

TABLE IV

Stability of Lacistorhynchus urease activity

Material	Holding time (hours)	Holding temperature			
		0° C.		20° C.	
		Tissue activity	Specific activity	Tissue activity	Specific activity
Intact worms	Freshly collected	3450	45.6	—	—
	24	3558	45.6	—	—
	84	3568	45.1	—	—
	110	3336	41.7	—	—
Homogenates	0	3689	43.4	3689	43.4
	1	2329	27.4	1862	21.9
	2	1785	21.0	1360	16.0
	4	1292	15.2	799	9.4
	8	765	9.0	604	7.1
	16	587	6.9	408	4.8
	24	323	3.8	255	3.0

Activities are the average of duplicate determinations from one-minute incubations at 20° C.

C. *Activity exhibited by intact worms*

A single experiment was designed to demonstrate urease activity in intact *Lacistorhynchus*.

One hundred freshly collected worms were divided into four lots of 25 worms each and given a preliminary incubation of three hours at 20° C. in urea-free saline as previously described.

Experimental incubations were performed in respirometer vessels with two sidearms. The main compartment of each vessel contained 1.0 ml. saline with 300 m*M* [^{14}C] urea (California Foundation for Biochemical Research) having a specific activity of 6.67×10^{-3} microcuries per micromole. The center well of each vessel contained 200 microliters 30% KOH for CO_2 capture; sidearm 1 contained 400 microliters of a 1 : 1 (v/v) mixture of 20% TCA and 0.5 *M* lactic acid.

Sidearm apertures were closed with greased, ground-glass stoppers. A worm lot was introduced into the main compartment of each vessel and the mouth of the vessel was immediately closed with a greased rubber stopper, further secured with a rubber band.

TABLE V

Recovery of ammonia and $^{14}CO_2$ following incubation of intact Lacistorhynchus in [^{14}C] urea

Worm group	Wet weight (mgm.)	Acid tip-in (minutes)	Ammonia recovered (micromoles/gm. wet tissue)	$BaCO_3$	
				Mgm.	Radioactivity (counts/min./mgm./gm. wet tissue)
1	155.6	0	11.6	3.3	7,352
2	186.0	5	52.7	3.7	34,559
3	183.2	10	71.0	4.0	39,885
4	170.6	20	102.1	4.6	41,882

See text for experimental details.

The vessels were incubated in a water bath at 20° C. and the acid mixture was tipped in and thoroughly mixed with the contents of the main compartment at desired times. The vessels were allowed to stand 10 hours at room temperature for CO_2 capture, following which period they were opened and the KOH removed from the center well; the latter was rinsed with ten 200-microliter portions of water and these were added to the KOH solution.

For ammonia capture 200 microliters 1 N H_2SO_4 were placed in sidearm 2. One milliliter of a saturated solution of K_2CO_3 was added to the main compartment, the vessels were again sealed, and 24 hours were allowed for diffusion of the ammonia. The H_2SO_4 was removed together with five 200-microliter rinses of the sidearm, diluted to 10.0 ml. and portions were subjected to microdiffusion and ammonia determination as previously described.

Carbon dioxide was precipitated from the KOH solution by the addition of 3.0 ml. of 0.1 N (each) $BaCl_2$-NH_4Cl. The precipitated $BaCO_3$ was centrifuged down at low speed and twice washed with water, and finally plated as a slurry in 70% ethanol on tared stainless steel sample pans (Nuclear-Chicago, SC-12). Radioactivity was determined with a gas flow counter (Nuclear-Chicago, D-47, with "Micromil" window).

Examination of the data obtained, presented in Table V, clearly indicates the production of ammonia and $^{14}CO_2$ from [^{14}C] urea by intact *Lacistorhynchus*. Significant radioactivity of $BaCO_3$ from the sample in which the acid was tipped in at zero-time indicates that the acid mixture is not instantaneously lethal.

The ammonia captured following five minutes' incubation is equivalent to hydrolysis of 316 micromoles urea per gram wet tissue per hour, or about one-tenth the tissue activity of homogenate preparations (Table I).

Because of the high urea concentration employed in the experiment, urea disappearance could not be measured by the Archibald (1945) method; hence no attempt was made to relate urea disappearance and ammonia and $^{14}CO_2$ production.

Discussion

Considerable scepticism has attended the periodic reports of urease in lower animals. This has probably been in part due to the low activities recorded as well as to the inadequacy of experimental procedures used in some instances. Too, doubt regarding the validity of observations on animal urease has probably been strengthened by the fact that activities attributed on numerous occasions to gastrointestinal mucosae are, in fact, of bacterial origin (see Kornberg and Davies, 1955).

In any event, Sumner (1951) offered the opinion that the entire question of animal urease needs reinvestigation, an opinion reiterated by Cohen and Brown (1960) in a more recent review.

The quantity of activity exhibited by the tapeworm urease reported here is of considerable interest. In Table VI the present author has assembled data from a number of other reports of urease of invertebrate origin and compared these with jackbean activity. It is to be understood that because of the widely varying conditions under which these assays were performed, Table VI, of necessity, represents only approximate comparisons. Dry weight values were estimated to be 20% of wet tissue weights when only the latter were available.

It is easily seen that the tissue activity of the tapeworm urease herein reported compares favorably with that of the jackbean material.

The use of antibiotics or other procedures designed to insure bacteriostatic conditions was not resorted to in the present study. Since tapeworms lack a gut, microorganisms would only be present as surface contaminants. It is highly unlikely that the urease activities observed with *Lacistorhynchus* can possibly be attributed to microorganisms. If they were indeed due to surface contaminants, then one might reasonably expect activity to be exhibited in tissue preparations of *Calliobothrium*, a worm parasitizing the same host as *Lacistorhynchus* (Table I). Too, while living worms retain full activity for a considerable period of time, tissue preparations rapidly lose activity, which would be most improbable were the activity of microbial origin (Table IV).

Of great interest to students of parasitism is the fact that cestodes seem to have developed more than one method of adapting to the enormous physiological uremia of their elasmobranch hosts, a condition which Smith (1936) believes to be an archaic biochemical property of this vertebrate class. Among tetraphyllids, urea is apparently not metabolized, but rather is an important osmotic constituent of these worms. Urease activity does not seem to be ubiquitous among trypanorhynchs (Table I), and may not even be characteristic of smaller taxonomic groups within

the Trypanorhyncha, since Dollfus (1942) assigns *Lacistorhynchus* and *Grillotia* to the same family.

Although most previous evidence indicates that adult tapeworms are poikilosmotic animals, consideration of possible osmo-regulation must be given to those forms inhabiting elasmobranch hosts and metabolizing urea (see Read and Simmons, in press).

There are, as yet, no data clarifying the biochemical consequences of urea hydrolysis by *Lacistorhynchus*. Although the hydrolysis of urea is an exergonic reaction, it has never been demonstrated that the free energy available can be trapped for useful work (Cohen and Brown, 1960). The possibility that *Lacisto-*

TABLE VI

Estimated urease activities of various invertebrate species compared with jackbean

Source	Tissue tested	Activity (mgm. urea hydrolyzed per gm. dry weight per 5 mins.)	Temperature (degrees Centigrade)	pH	Approximate concentration of urea (molar)	Author
Jackbean	Meal (not defatted)	486–735	20	7.0	0.250	Damodaran and Sivaramakrishnan (1937)
Cestodes						
Lacistorhynchus tenuis	Whole	70	20	7.0	0.400	Present
Pterobothrium lintoni	Whole	700	20	7.0	0.400	Present
Nematodes						
Ascaris lumbricoides	Intestine	0.73	?	6.0	0.007	Savel (1955)
	Muscle	0.28	?	6.0	0.007	Savel (1955)
Nematodirus spp.	Whole	0.036	?	7.3	0.008	Rogers (1952)
Sipunculid						
Sipunculus nudus	Intestine	0.30	?	?	0.0013	Florkin and Duchateau (1942)
Annelid						
"*Lumbricus agricola*"	Whole	0.0007	?	?	0.167	Przylecki (1922)
Molluscs						
Mytilus edulis	Whole	0.009	?	?	0.167	Przylecki (1922)
Helix pomatia	Whole	0.014	?	?	0.167	Przylecki (1922)
Arthropods						
Lucilia sericata	Maggots reared aseptically	1.2	35–36	7.4	?	Robinson and Baker (1939)
Astacus fluviatilis	Whole	0.001	?	?	0.167	Przylecki (1922)

rhynchus might further utilize at least part of the ammonia and carbon dioxide produced by its urease must be considered. Campbell (1960) demonstrated the formation of α-[1-^{14}C] alanine and, probably, [^{14}C] succinic acid from the degradation of [2-^{14}C] uracil by the rat cestode, *Hymenolepis diminuta*, for which the most tenable explanation was a mechanism of carbon dioxide fixation; Daugherty (1954) has reported the synthesis of amino nitrogen from ammonium carbonate by the same species.

The author expresses appreciation to the Supply Department of the Marine Biological Laboratory for providing fishes. He is very grateful to Dr. Clark P. Read for encouraging the work, for supporting it, and for criticizing the manuscript, and to Dr. J. Wayne Campbell for providing certain facilities and for technical advice.

Summary

1. Strong urease activity was measured in tissue preparations of two species of trypanorhynch cestodes; activity was not detected in seven species of tetraphyllids, nor in a third trypanorhynch.

2. Tissue activity of *Lacistorhynchus tenuis* homogenates was in excess of 3000 micromoles urea hydrolyzed per gram wet tissue per hour; that of *Pterobothrium lintoni* was about ten times greater. Tissue activity of intact *Lacistorhynchus* was about one-tenth that of homogenate preparations.

3. The worm activity was partially characterized in homogenate preparations.

4. Living worms retain full activity for at least 110 hours, but homogenates rapidly decline in activity; less than 10% of the original activity is measured after 24 hours.

5. Production of ammonia and of $^{14}CO_2$ from [^{14}C] urea was demonstrated using intact *Lacistorhynchus*.

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TRYPTOPHAN PYRROLASE ACTIVITY IN THE LIVER OF ADULT RANA PIPIENS [1]

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It has been shown by Knox and Mehler (1950) that the activity of mammalian liver tryptophan pyrrolase (peroxidase-oxidase) was greatly increased after injection of tryptophan. The enzyme has been found in the livers of a variety of mammals but not in other tissues (Knox, 1955). Knox and Mehler (1951) further demonstrated that this induction of increased enzyme activity was not controlled by substrate alone. Increased liver enzyme levels could be produced by various stressing conditions, such as section of the spinal cord (Thompson and Mikuta, 1954), and by injections of such substances as histidine and tyrosine (Knox and Mehler, 1951) and histamine and adrenalin (Knox, 1951) in intact animals. In adrenalectomized animals liver enzyme activity could be increased by injections of adrenocorticotropic hormone (Geschwind and Li, 1953) and the glucocorticoid hormones (Thompson and Mikuta, 1954; Knox and Auerbach, 1955). Knox and Auerbach (1955) concluded that there were probably two types of inducing agents, the substrate and an adrenal hormone, and that the enzyme could be induced in adrenalectomized rats by the substrate independently of the hormone. Civen and Knox (1959) further demonstrated that the hormonal induction was independent of substrate concentration and not due to an increase in the level of tryptophan in the tissues produced by the glucocorticoid tissues. More recently Feigelson and Greengard (1961) have demonstrated that rat liver tryptophan pyrrolase is activated by an iron protoporphyrin located in the microsomes.

In the course of investigations on the synthesis of tryptophan pyrrolase during the embryonic development (to be reported elsewhere) of *Rana pipiens* it was found that the controlling mechanisms of tryptophan pyrrolase activity in the frog livers are quite different from those of the adult mammal. It is the results of this investigation on the liver of adult *Rana pipiens* that are reported in the present paper.

Materials and Methods

Large adult male and female *Rana pipiens* were obtained from Vermont and maintained in running tap water at 12–18° C. The procedure for assay of the enzyme was that described by Knox and Auerbach (1955) with two exceptions. The homogenate was prepared by grinding the liver in either a Teflon-Pyrex homogenizer [2] or a Virtis "23" homogenizer [3] for 2–3 minutes, and secondly the reactions were run in an air atmosphere. All other details were *exactly* as described by Knox and Auerbach. Under the conditions specified by these authors

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[2] Kontes Glass Co., Vineland, New Jersey.

[3] Virtis Company, Inc., Yonkers, New York.

enzyme activity was directly proportional to concentration of frog liver homogenate. The Knox and Auerbach procedure is based on the conversion of tryptophan to kynurenine which is the product actually measured during the assay. This conversion, however, is a two-step reaction, tryptophan pyrrolase catalyzing the first step, the conversion of tryptophan to formylkynurenine. The enzyme formylase, which catalyzes the conversion of formylkynurenine to kynurenine, the second step, is found in excess in mammalian liver homogenates and is not rate-limiting (Knox and Mehler, 1950). In all control and experimental frog livers, no reaction products absorbing at 321 mμ were detected. This indicated the absence of formylkynurenine, and formylase, therefore, is probably present in excess in frog livers and is not rate-limiting. L-tryptophan, L-histidine (free base), bovine adrenocorticotropic hormone (ACTH), cortisone acetate, and hydrocortisone acetate were obtained from the Nutritional Biochemical Laboratory, Cleveland, Ohio. Pituitaries were obtained by dissection from large adult females. All injections were given intraperitoneally. All enzyme activities are expressed in terms of μ moles of kynurenine formed per hour at 38° C., per gram dry weight of liver, ± standard error of the mean.

Results

During the early stages of this investigation adult females were used exclusively since it was our intention first to study the activity of the enzyme, under various

Table I

Basal activity of enzyme in liver of adult Rana pipiens females (non-ovulated) during the breeding season

Date	Number of animals	Activity*
4/27/60–5/3/60	10	11.59 ± 1.99
5/4/60–5/11/60	21	19.68 ± 2.20
5/18/60–5/23/60	9	25.60 + 2.62

* μ moles kynurenine formed per hour at 38° C., per gram dry weight of liver, ± standard error of the mean.

conditions, in the adult and ultimately to relate these findings to a study of the enzyme during embryonic development, starting with the ovarian egg. The results at first glance seemed to indicate that the enzyme activity in the female was extremely variable and that the frog was a rather poor experimental animal for this type of study. A more detailed analysis revealed that basal activity of the enzyme in the female was indeed variable but that the extreme variability was due to a progressive increase in enzyme activity following hibernation and during the breeding season. Table I summarizes these findings. It will be noted that a more than 100% increase in enzyme activity (11.59 ± 1.99 to 25.60 ± 2.62 μ moles kynurenine/hour/gm. dry weight of liver) occurred, in non-ovulated females, over an approximately 4-week period following hibernation.

A comparison of liver enzyme activity in females which had ovulated in nature with non-ovulated females revealed that sometime during or following the period of ovulation, enzyme activity radically decreased from a value of 25.10 ± 2.12 to 8.80 ± 1.00 (Table II).

It seemed probable that perhaps pituitary hormones were changing in activity

TABLE II

Basal activity of enzyme in liver of adult Rana pipiens ovulated females vs. non-ovulated females

State of ovulation	Number of animals	Activity*
Ovulated	17	8.80 ± 1.00
Non-ovulated	9	25.10 ± 2.12

* μ moles kynurenine formed per hour at 38° C., per gram dry weight of liver, ± standard error of the mean.

during the breeding season and affecting the enzyme activity. Accordingly pituitaries isolated from mature females were injected into mature ovulated and non-ovulated females. The results are summarized in Table III. In ovulated females the control activities were of the low pre-breeding season values. Injection of pituitary lowered the enzyme activity to 4.45 ± 0.161. That this activity-lowering effect of pituitary injection was not due to events either preceding or occurring simultaneously with ovulation is shown by the results of pituitary injections on non-ovulated females (Table III). In this case the non-ovulated females, after injection of pituitaries, ovulated, but the liver enzyme activity remained at a high level. Further consideration will be given to this point in the discussion section of this paper.

Since the results indicated that enzyme activity in the female varied to a considerable extent during the breeding season, subsequent experiments were carried out on the male frog. Comparable suppression of enzyme activity following pituitary injections was also encountered in the male. In fact, enzyme activity in the male was almost identical with that of the female after this treatment, 4.99 ± 0.619 vs. 4.45 ± 0.161 in the female (Table III). It was interesting to note that basal activity in the male, 9.64 ± 1.26 (Table IV), very closely approximated the basal activity in female livers after ovulation (8.80 ± 1.00, Table II).

Table IV summarizes liver enzyme activity in the male frog after injections of various compounds known to increase activity in the normal intact mammal.

TABLE III

Activity of enzyme in liver of adult Rana pipiens after pituitary injections

Number, sex, and state of ovulation of animals	Treatment	Time (hrs.) assayed after injection	Activity*
14, females, ovulated	2–4 pituitaries in 1 ml. of 10% Holtfreter's solution	5	4.45 ± 0.161
6, females, ovulated	1 ml. of 10% Holtfreter's solution	5	7.41 ± 1.18
6, females, non-ovulated before injection	2 pituitaries in 1 ml. of 10% Holtfreter's solution	18	24.16 ± 2.53
6, females, non-ovulated before injection	1 ml. of 10% Holtfreter's solution	18	22.36 ± 1.79
6, males	5 pituitaries in 1 ml. of 10% Holtfreter's solution	14⅔	4.99 ± 0.619
6, males	1 ml. of 10% Holtfreter's solution	14⅔	9.80 ± 0.41

* μ moles kynurenine formed per hour at 38° C., per gram dry weight of liver, ± standard error of the mean.

TABLE IV

Enzyme activity in liver of adult male Rana pipiens after various treatments

Number of animals	Treatment	Activity*
12	2 ml. L-histidine**	6.44 ± 0.66
12	2 ml. 0.65% NaCl	9.71 ± 0.97
6	ACTH***	7.56 ± 2.11
6	15% gelatin in 0.5% phenol****	9.65 ± 1.21
8	0.5 ml. cortisone acetate†	9.34 ± 1.00
8	0.5 ml. 0.65% NaCl††	9.59 ± 0.73
6	1 ml. hydrocortisone acetate†††	9.20 ± 0.98
6	1 ml. 0.65% NaCl	9.81 ± 1.47
6	3 ml. L-tryptophan††††	40.50 ± 4.90
6	3 ml. 0.65% NaCl	9.49 ± 0.53

* Micro moles kynurenine formed per hour at 38° C., per gram dry weight of liver, ± standard error of the mean. All animals sacrificed and livers assayed 4–6 hours after the last injection.

** Six hundred and thirty mg. in 12 ml. of 0.65% NaCl.

*** One-quarter ml. (8 units/ml. of 15% gelatin in 0.5% phenol); 0.1 ml. 24 hours later; then 0.25 ml. 16 hours later.

**** One-quarter ml.; 0.1 ml. 24 hours later; then 0.25 ml. 16 hours later.

† Ten mg./4 ml. of 0.65% NaCl; for 3 days at 24-hour intervals.

†† For 3 days at 24-hour intervals.

††† Eighty mg./10 ml. 0.65% NaCl.

†††† Seventy-five mg./15 ml. of 0.65% NaCl.

Injections of L-tryptophan produced a 427% increase in enzyme activity. Rather surprisingly, injection of the adrenal cortical hormones, cortisone acetate and hydrocortisone acetate, produced no significant change in enzyme activity in contrast to the increases in activity found in the mammal after their injection. Similarly, L-histidine and ACTH produced no increase as they do when injected into the mammal, and in fact the results suggest that these substances may produce an inhibition instead of an augmentation.

DISCUSSION

The observation that liver tryptophan pyrrolase activity increases in the female frog during the breeding season, to our knowledge, has no counterpart in the recorded observations of activity in the female mammal. Since no investigation, to our knowledge, has been carried out in the female mammal to check tryptophan pyrrolase activity during the estrous cycle it would be presumptuous to state that the amphibian, as exemplified by the frog, is in direct contrast to the mammal with respect to this observation. It is possible that a comparable investigation carried out in the mammal would lead to similar results. The mechanism of this increase remains to be elucidated.

It is, however, well known that in female frogs maintained at temperatures above 15–18° C. for several weeks there is a rapid consumption of the fat bodies and even of the eggs within the ovaries (Rugh, 1935). If female frogs are maintained at 20–25° C. for prolonged periods, abnormal eggs are produced after ovulation. It is possible that the ovarian eggs and fat bodies of female frogs which have not ovulated early in the breeding season begin to degenerate at the relatively warmer temperatures prevalent during the latter part of the season.

Concomitant with this degeneration one could assume a release of tryptophan (from the fat bodies and eggs into the maternal circulation) which is eventually transported to the liver where a tryptophan induction of enzyme activity would occur. While no figures are available, it is our observation that towards the end of the breeding season the percentage of non-ovulated females furnishing 95–100% fertilizable and normally developing eggs diminishes sharply. The return to relatively low enzyme activity following ovulation would offer partial support to this hypothesis. The failure of pituitary injections in non-ovulated females (although bringing about ovulation in these animals) to lower the enzyme activity would require under this hypothesis that the blood tryptophan concentration was sufficiently high to maintain appreciable enzyme activity (or continued induction) for some time after ovulation had occurred. Two experiments carried out in this laboratory indicate that one week after ovulation had been induced by pituitary injection, and the females stripped, the enzyme activity had dropped to its pre-breeding season value. Enzyme activity for these experiments was 8.90 μ moles of kynurenine per hour, per gm. dry weight of liver. Measurements of ovarian and fat body vs. blood tryptophan concentrations during the breeding season would be of value in testing the validity of this hypothesis.

It has been demonstrated by Sims and Bishop (1947) that the pituitary gland of *Rana pipiens* does undergo seasonal variations, increasing in gonadotropic potency between January and March. The possibility that pituitary hormones are increasing during the breeding season and affecting the adrenal cortical hormone balance, which in turn brings about an increase in enzyme activity, seems to be negated by the decrease in activity following pituitary injection. It is implicit in the hypothesis that the addition of pituitary should either augment enzyme activity or have no effect if the synthesis of enzyme is going on at a maximum rate before injection of pituitary. The most probable explanation for the pituitary effect is that the decrease of activity is due to growth hormone, for it has been demonstrated by Wood and Knox (1954) that growth hormone when injected into the mouse brings about a decrease in liver enzyme activity. Estrogenic hormones undoubtedly vary in concentration during the breeding season and should be tested for their effect on enzyme activity.

A third explanation for the increase in enzyme activity during the breeding season remains to be considered. It is possible that the feeding habits of the adult female are extremely variable and it is conceivable that the female upon breaking hibernation consumes a diet rich in tryptophan, leading to an induction of enzyme activity. Following ovulation the consuming of a diet low in tryptophan would in turn lead to a decrease in enzyme activity. A careful search of the literature has revealed no information as to the variation in the food habits of the female frog during this period of the year.

Recently Feigelson and Greengard (1961) have demonstrated that rat liver tryptophan pyrrolase is activated by an iron protoporphyrin present in liver microsomes. It has been further demonstrated (Greengard and Feigelson, 1961) that tryptophan treatment caused an approximately 20% increase in the iron protoporphyrin concentration of liver microsomes, and that the initial rise in enzyme activity after tryptophan treatment was due to an increased degree of saturation of the enzyme with respect to the activator. It is possible that the increases

noted in the enzyme activity of frog liver homogenates during the breeding season and following tryptophan administration and the suppression of activity following pituitary injection are due to changes in the iron protoporphyrin content of liver microsomes. Experiments carried out on the frog which are comparable to those of Feigelson and Greengard will elucidate the role of this activator under the conditions mentioned above.

The failure of the adrenal cortical hormones and of ACTH and L-histidine to produce an augmentation of enzyme activity is in direct contrast to the increases in activity found in the mammal after similar treatment (Knox and Mehler, 1951; Geschwind and Li, 1953). The data for ACTH and L-histidine suggest that these substances may in fact bring about a decrease in activity but further experiments are needed to substantiate this possibility. Although Carstensen *et al.* (1961) have demonstrated the production of aldosterone and corticosterone by *Rana catesbiana* adrenal tissue under the stimulation of bovine ACTH, there appears to be no published information regarding the adrenal cortical hormones in *Rana pipiens*, and it is possible that the amphibian cortical hormones are quite different from those of the mammal. Injections of frog adrenal homogenates would be extremely helpful in establishing this hypothesis.

The frog appears to be an ideal organism for studying the mechanism of substrate induction in metazoa of this enzyme, for the results are not complicated by the possibility of an effect being mediated through the adrenal.

Summary

1. The effect of the injection of L-tryptophan, L-histidine, ACTH, cortisone acetate, hydrocortisone acetate, and pituitary on liver tryptophan pyrrolase activity was studied in adult male and female *Rana pipiens*.

2. L-tryptophan produced a 427% increase in activity but cortisone acetate and hydrocortisone acetate injections had no effect. ACTH and L-histidine injections produced a slight decrease in activity. Pituitary injections brought about a marked decrease in activity.

3. In adult females there was a progressive increase in enzyme activity following hibernation and during the breeding season. This progressive increase was followed by a sharp drop in activity following ovulation.

4. The results are discussed in terms of the mammalian data.

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POLYPHENOL OXIDASE IN THE TEGUMENTAL GLANDS IN RELATION TO THE MOLTING CYCLE OF THE ISOPOD CRUSTACEAN ARMADILLIDIUM VULGARE[1]

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Various hypotheses have been advanced to explain the function of the tegumental glands of Crustacea. The suggestions by early workers that they might be salivary glands, slime glands, sense organs, or excretory organs have been reviewed and disposed of by Yonge (1924, 1932), who believed that they might secrete the epicuticle. (Yonge called this layer the cuticle, but in this paper the terminology adopted by Richards, 1951, will be employed. According to this terminology, the outer, non-chitinous layer of the cuticle is the epicuticle and the inner chitinous layer is the procuticle.) Drach (1939), on the other hand, thought the epidermis secretes the epicuticle. Arguments for and against Yonge's view have been reviewed by Dennell (1947b, 1960), who suggested that since the tegumental glands are most abundant where the cuticle is tanned the most, they may produce the polyphenol oxidase involved in tanning.

This suggestion by Dennell has been investigated by Krishnan (1951). He used the nadi reagent as a test for oxidase and obtained a positive reaction in the tegumental glands of *Carcinus,* their ducts, and the pigment layer of the cuticle during middle and late intermolt when melanin is forming in the pigment layer. The reaction was thermo-labile and cyanide-sensitive. Also the glands and their ducts became dark brown in catechol during this same period. He concluded the tegumental glands secrete polyphenol oxidase for melanin production in the cuticle. Whether they also secrete polyphenol oxidase earlier for tanning is not known. Apparently Krishnan did not study this earlier stage in the molting cycle.

In order to discover whether the tegumental glands secrete polyphenol oxidase for tanning, the molting cycle of the pillbug, *Armadillidium vulgare,* has been studied and polyphenol oxidase has been looked for in the tegumental glands of the legs at every stage of the molting cycle.

Materials and Methods

Several hundred specimens of *Armadillidium vulgare* collected in a garden in Seattle, Washington, were maintained in the laboratory in large jars partly filled with moist dirt. Pieces of carrot and potato were placed in each jar for food.

Tegumental glands were obtained for study by dissection of pereiopods in 0.35 *M* NaCl. A separate leg was removed from a living animal for each test and used immediately. The glands of one segment were tested with the reagent and

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the glands of another segment of the same leg were tested with the reagent plus inhibitor. The glands are very abundant in the carpus and merus, less abundant in the ischium, and small and scarce in the basis. They seem to be absent from the propus and dactylus. To ensure uniformity, only animals in molting stage C were used for the tests for polyphenol oxidase. The dissections were made in 0.35 *M* NaCl because this concentration has a freezing point depression close to 1.18°, the average value for *Armadillidium* according to Parry (1953). No visible changes in the glands take place in this solution, whereas the secretory droplets in the glands burst in hypotonic solutions.

The nadi reagent was prepared immediately before use by mixing 0.1 ml. of 1% a-naphthol in 40% ethanol and 0.1 ml. of 1% N,N-dimethyl-p-phenylenediamine hydrocholoride with 2.5 ml. of buffer. Acetate, phosphate, carbonate, and glycine buffers were used to provide a range in pH from 4 to 11.

A stock of *Armadillidium vulgare* adults was also maintained, one animal to a jar, in 100-ml. jars, each having a shallow layer of moist dirt and potato and carrot for food. By making daily observations on each animal, criteria for recognizing the different molting stages were developed, as described below.

Results

Molting stages. The stage designations developed by Drach (1939) for the Decapoda and some of the modifications of these designations developed by Charniaux-Legrand (1951) for the Amphipoda have been used.

The half of the body posterior to the suture separating the fourth and fifth free thoracic segments molts first, and the half of the body anterior to this suture molts a few days later. Since the molting stages of the two halves of the body can be determined independently, one can designate the molting stage of an animal by stating the stages of each half; for example, anterior: stage C^a, posterior: stage $D_1{}^p$. The letters a and p indicate anterior and posterior.

The following descriptions apply to either half.

Stage A. Immediately after the molt. The body half appears dark brown or black, and the cuticle is soft, shiny, and slightly sticky. Grains of dirt may adhere to it. The legs are not functional, but the animal can walk with the legs of the other half of the body. At first the half in stage A is only about half as long as the other half, but it begins to expand immediately. At the end of stage A, expansion is completed, the cuticle is becoming gray in color, and the legs are beginning to be functional. Duration of stage A: 1–2 hours.

Stage B. The cuticle is gray in color. It has only begun to harden; the terga can be depressed without cracking. The body half has expanded to its full, new size. The pereiopods are functional but their cuticle, as measured in optical section, has not yet reached its definitive thickness (Fig. 1). The animal normally eats its exuviae during this stage. Duration of stage B: several hours.

Stage C. The body has achieved its definitive coloration and hardness. In the early part of this stage the cuticle of the pereiopods becomes progressively harder, beginning distally. Stage C could be subdivided in terms of this progressive hardening. At the same time the cuticle of the pereiopods becomes progressively thicker (compare Figs. 1 and 2). Duration of stage C: about 15–60 days.

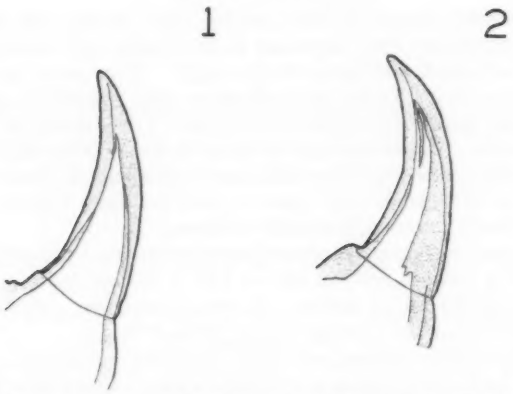

FIGURE 1. Tip of left first pereiopod in stage B. 280 ×.
FIGURE 2. Tip of right first pereiopod of same specimen 10 days later. Stage C.

Stage D. Preparation for the molt. This stage can conveniently be subdivided, as follows:

D_1. New claws begin to form in the pereiopods, as described by Charniaux-Legrand (1951) in amphipods (Fig. 3). While the posterior half of the body is in stage D_1 a pair of white plates develops on each of thoracic sterna 1 to 4. Duration of stage D_1: a few days to a week.

D_2. The new claws have become amber-colored, and the pre-exuvial layers of the new cuticle are secreted over the entire body half. While the anterior half of the body is in stage D_2 the white sternal plates disappear. Duration of stage D_2: about one day.

D_3. As in amphipods, nothing corresponding to stage D_3 of decapods is readily discernible in *Armadillidium*.

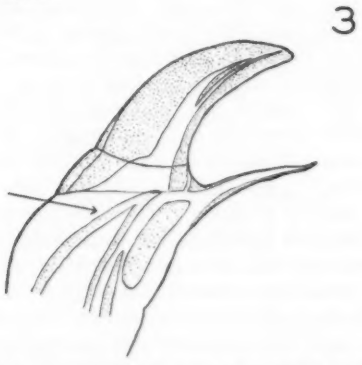

FIGURE 3. Tip of right first pereiopod in stage D_1. The tip of the new dactylus (arrow) can be seen inside the old. The leg was too opaque for the proximal part of the new dactylus to be seen.

D_4. The body half can be compressed easily with the fingers, and the old cuticle can be flaked off. Duration of stage D_4: a few hours.

Stage E. Ecdysis. Molting may occur at any hour, day or night.

Nadi test at different stages of the molting cycle. The tegumental glands of the legs were tested with the nadi reagent in phosphate buffer at pH 7.4 in every stage of the molting cycle and at different intervals within stages C and D. In every case, deep blue color developed in the secretory droplets within a few minutes in the absence but not in the presence of 0.002 *M* NaCN.

Additional tests for polyphenol oxidase. In addition to NaCN, two other inhibitors, NaN_3 and salicylaldoxime, were used with the nadi reagent, and the effect of heat was tested. According to Keilin (1936), catechol oxidase (polyphenol oxidase) from mushrooms was not inhibited by 0.002 *M* NaN_3 at pH 7.3, but 68% inhibition was obtained at pH 5.9. On the other hand only 0.001 *M* NaN_3 produced 80–90% inhibition of cytochrome oxidase at pH 6.5–7.5. In order to test for this same kind of behavior by the oxidase of the tegumental glands,

TABLE I

Results of nadi tests on tegumental glands at different pHs

Without inhibitor

pH	4	5	6	6.8	7	7.2	7.4	8	9	10	11
	−	−	+	+	+	+	+	+	+	±	±

With 0.02 *M* NaN_3

pH	5	6	6.8	7	7.2	7.4	8	9	10	11
	−	−	−	−	+	+	+	+	±	−

the nadi reagent in 0.002 *M* NaN_3 was used in buffers over a pH range from 4 to 11. For comparison with cytochrome oxidase, the nadi-positive granules of the leg muscles of *Armadillidium* were used. The two tissues could be examined together on the same slide. It was found that 0.002 *M* NaN_3 completely prevented the development of blue color in the granules of the muscles throughout the entire range, pH 6 to pH 8, in which indophenol blue developed in muscles in the control slides. The same concentration of NaN_3 had no discernible effect on the reaction in the tegumental glands at any pH, however. Accordingly, the series was repeated using 0.02 *M* NaN_3. This time, inhibition was obtained in the secretory droplets of the tegumental glands below pH 7.2, as shown in Table I. Without azide, blue color developed in all tests from pH 6 to pH 9. At pH 10 and pH 11, blue color sometimes developed in some of the glands. With 0.02 *M* NaN_3, no blue color developed at pH 5, 6, 6.8, or 7, and normal blue color developed as rapidly as in the controls at pH 7.2, 7.4, 8, and 9. Blue color sometimes developed in some of the glands at pH 10, never at pH 11. A pH of 4 was not tested.

Salicylaldoxime is a reagent for copper and therefore should inhibit the copper-containing enzyme polyphenol oxidase. The tegumental glands were tested with

the nadi reagent with and without 0.001 *M* salicylaldoxime and with and without 0.01 *M* salicylaldoxime. At 0.001 *M*, blue color developed about half as rapidly (14–15 minutes) as in the controls (5–6 minutes). At 0.01 *M*, no color developed at all.

The nadi test was also negative in glands that had been incubated at 65° C. for 30 minutes.

Fresh tegumental glands were also treated with 1% 3,4 DL dihydroxyphenylalanine (dopa) in phosphate buffer, pH 6.8. After three hours at 22° C., the secretory droplets were black. None of the other tissues had become colored.

Discussion

Studies on molting and related phenomena in crustaceans are sometimes hampered by the difficulty of obtaining the desired stages in the molting cycle when needed. By using *Armadillidium* and the criteria for recognizing the different stages as described in this paper, however, one can obtain any stage at any time. For instance, in a stock of only 60 animals an average of one will molt each day. To designate short periods within each of the primary stages, substages or number of hours before or after an event can be used. George and Sheard (1954) have used the latter method in their description of changes in the epidermis and cuticle of *Porcellio scaber*.

From the evidence presented in this paper, the tegumental glands seem definitely to contain polyphenol oxidase. The nadi test is rather uncertain and variable—poor staining was obtained if the stock solutions were more than a month or less than a week old! But inhibition of the reaction by cyanide, azide, salicylaldoxime, and heat clearly indicates the presence of an enzyme. Inhibition by azide was exactly as predicted from Keilin's work with catechol oxidase of mushrooms, except that a higher concentration of inhibitor was needed.

The most specific test for polyphenol oxidase used was treatment with dopa, which is oxidized to dopa quinone by this enzyme. The dopa quinone then becomes melanin by polymerization.

The tegumental glands of the legs of *Armadillidium* are similar histologically to tegumental glands of other parts of the body and to tegumental glands of other crustaceans. Their function is probably to secrete polyphenol oxidase into the epicuticle from where it diffuses inward and catalyzes sclerotization in the epicuticle and exocuticle (Dennel, 1947b). The evidence for this is three-fold. First, the glands develop and degenerate with each molting cycle, and the peak in their activity occurs some time before molting (Yonge, 1932; Gorvett, 1946). It may be that the peak coincides with the appearance of polyphenol oxidase in the epicuticle, but further work will have to be done to determine whether this is the case. Second, the ducts of the glands extend to or through the epicuticle, and these ducts are most abundant where the cuticle is tanned the most (Dennell, 1947b, 1960). The presence of the ducts extending through the procuticle to the epicuticle is significant because the epicuticle is the part of the cuticle where polyphenol oxidase appears first (Dennell, 1947b). However, in the insect *Sarcophaga*, in which the enzyme also appears first in the epicuticle, there is evidence that it originates in the epidermis, diffuses through the procuticle, and somehow accumulates in the epicuticle (Dennell, 1947a). Third, as demonstrated in this paper, the tegu-

mental glands do contain polyphenol oxidase. In addition to this evidence, direct evidence that the glands release polyphenol oxidase into the epicuticle at or before the time when it is used in tanning would be desirable before one concluded that they do so. It may be that the enzyme is involved in the metabolism of the glands and that the secretory product is some other material. Both mucus (Farkas, 1927) and lipid (Yonge, 1932) have been reported in tegumental glands, and there is evidence that tyrosinase (polyphenol oxidase) may function as a terminal oxidase in respiration, at least in plants and insects (Dawson and Tarpley, 1951; Patterson, 1958).

The presence of polyphenol oxidase in the tegumental glands of the legs of *Armadillidium* throughout the molting cycle is not inconsistent with the idea that the enzyme is released at a particular time. The enzyme may be in storage most of the time. However, it raises the question of why *Carcinus* is different in this respect. Krishnan found polyphenol oxidase only during middle and late intermolt in *Carcinus*. Another difference in *Carcinus* is that the tegumental glands also apparently secrete polyphenol oxidase into the pigment layer, where it catalyzes melanin production (Krishnan, 1951). Presumably this does not occur in *Armadillidium*, which lacks a pigment layer.

Undoubtedly some of the different types of tegumental glands perform different functions. Gorvett (1946) has described six types in isopods, and he presents evidence that the lobed glands of the uropods and lateral plates secrete bad-tasting and bad-smelling material that protects against spiders (Gorvett, 1951, 1952, 1956). Other types may have yet different functions.

Summary

1. Criteria are presented for distinguishing the molting stages of *Armadillidium vulgare*.

2. An oxidase is present in secretory droplets in the tegumental glands of the legs throughout the entire molting cycle.

3. This oxidase seems to be the polyphenol oxidase involved in sclerotization of the cuticle.

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SEASONAL VARIATIONS IN O_2-CONSUMPTION OF UCA PUGNAX

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Within the past few years studies on O_2-consumption of the fiddler crab, *Uca pugnax,* have provided descriptions of diurnal and lunar-tidal rhythms (Brown, Bennett and Webb, 1954). Webb and Brown (1958) have described the persistence of lunar-tidal rhythms of O_2-consumption for as long as three weeks in the laboratory. Both of these investigations were carried out during the summer months when these animals regularly leave their burrows at the time of low tide. Because of the marked stability exhibited by the lunar-tidal rhythm and the fact that the period of the rhythm is not one normally associated with human activity in the laboratory, it was of interest to study this rhythm at various times of year. We were particularly concerned with two aspects: (1) any seasonal variations in either the diurnal or the lunar-tidal rhythms, and (2) the degree of interdependence between the diurnal rhythm and the lunar-tidal one as indicated, for example, by the extent to which a change in one occurred simultaneously with a change in the other.

Materials and Methods

These experiments were carried out at the Marine Biological Laboratory at Woods Hole, Mass., and the animals used were collected at Chappaquoit Beach, or at Silver Beach, both on Cape Cod, Mass. During the summer months collections were made at approximately weekly intervals, the animals being picked up as they ran on the beach near the time of low tide. All of these collections were made at Chappaquoit Beach, the last one in 1959 on September 30. On January 7, 1960, and again on January 29, animals were obtained from the Supply Department of the Marine Biological Laboratory. On both of these occasions the animals were dug from their burrows at Silver Beach, which is about one mile from Chappaquoit and where low tide occurs within a few minutes of its occurrence at Chappaquoit. In the laboratory the animals were kept in large white enamel pans with a small amount of sea water which was changed daily. Only males, of the species *Uca pugnax,* ranging in weight from 2 to 4 grams were used. The animals were kept in a laboratory where they were exposed to the normal diurnal changes in illumination, and thus they experienced decreasing photoperiods until late December, and in January and February, an increasing one.

Oxygen consumption was measured by means of Brown respirometers (Brown, 1957) with the conditions the same as those described by Webb and Brown (1958). A maximum of six barostats was used at any one time. Thus the maximum num-

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Northwestern University, NONR-122803.

ber of animals represented on any one day is 24, and the minimum is four (one barostat). Throughout most of the study three barostats (12 animals) were being used. The lever system was such that an excursion of 1.4 mm. on the record was equivalent to 1 ml. of O_2 consumed. Animals usually remained in the respirometers three or four days. Only complete days of data were used; thus the portions of records obtained between the time of setting up the respirometers and the first midnight were excluded, as were the terminal portions from midnight of the last day of each run.

Results

Data are available for 11 semi-monthly periods from July 25, 1959, to February 18, 1960, and for two such periods from June 25 to July 25, 1960. The mean values of O_2-consumption and the ranges for the mean cycles are presented in

Table I

Respiratory rates and cycle ranges for U. pugnax

Calendar period	Mean rate (ml./kg./hour)	Diurnal range (% of mean rate)	Lunar range (% of mean rate)
A. 7/25– 8/8	81	40%	24%
B. 8/9 – 8/24	80	33%	26%
C. 8/24– 9/8	80	28%	31%
D. 9/8 – 9/22	70	36%	37%
E. 9/22–10/6	63	28%	48%
F. 10/11–10/25	60	43%	22%
G. 10/25–11/8	48	34%	20%
H. 11/19–12/2	43	33%	22%
I. 12/4 –12/18	44	28%	27%
J. 1/9 – 1/23	46	36%	33%
K. 2/4 – 2/18	40	31%	21%
L. 6/25– 7/9	77	33%	24%
M. 7/10– 7/25	86	31%	27%

Table I. It is evident that there occurs a marked reduction in rate of respiration throughout the autumn season. The values can be roughly separated into three categories: June 25 through September 8, when the values are highest and quite constant; September 8 through November 8, when a decreasing series is obtained; November 8 through February 18, during which time the values show little or no change and are low.

It is also clear from Table I that the range of a 15-day mean cycle bears no consistent relationship to the mean hourly rate or to the season of the year. Practically the entire gamut of fluctuations in range of the solar-day cycle is encompassed within the first three periods during which the mean rate remains essentially unchanged.

In Figure 1 is seen a series of solar-day curves, each calculated for a 15-day period, plotted as deviations from the mean for the period. The periods are those recorded in Table I. It can be seen that throughout the series the form of the solar-day curve remains relatively stable. It exhibits the highest values during the

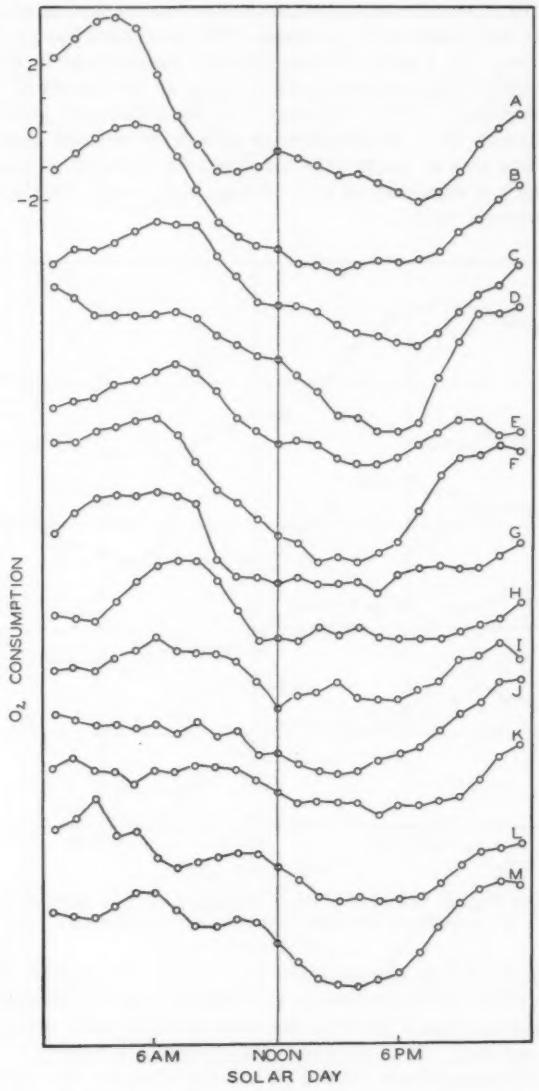

FIGURE 1. Mean diurnal variations in O_2-consumption of *Uca pugnax* for 13 15-day periods during 1959–60. The curves are for the periods appearing in Table I where each period is identified by the same letter as the corresponding curve in the Figure.

hours between 10 P.M. and noon, and the lowest values during afternoon or early evening. There are variations in position of the maximum but no obvious pattern to these variations. In Figure 2 these data are summarized in the form of four curves, one for each of the following periods: July 25 to October 25, 1959; October 25, 1959, to January 23, 1960; February 2 to 18, 1960; and June 25 to July 25, 1960. It is obvious that the absolute amplitude is reduced during the winter months but as was seen in Table I the absolute rate is decreased also. Thus when amplitude (range) is expressed as a percentage of the mean for the period, there is no consistent seasonal trend.

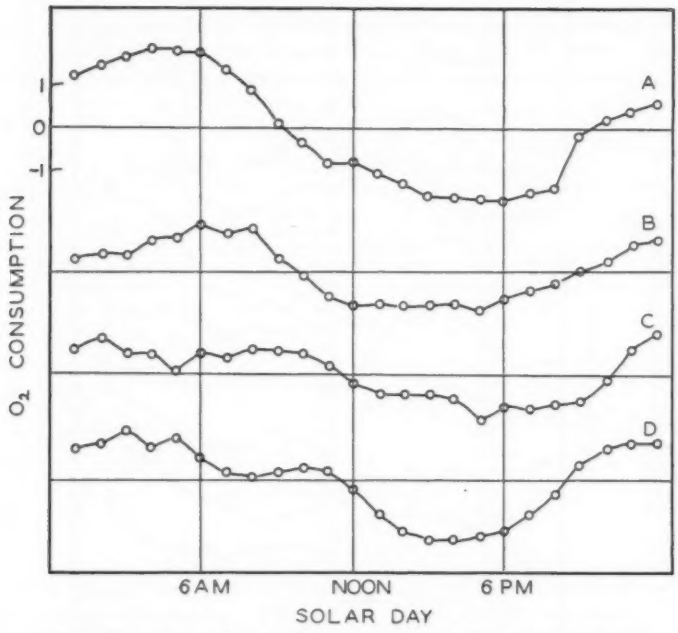

FIGURE 2. Mean diurnal variation in O_2-consumption of *Uca pugnax* for four periods throughout the year (see text for explanation).

When 15 days of data are analyzed for a lunar-day rhythm and each day is used only once, there will be present in the resulting curve a residual of the diurnal rhythm since any particular point of the solar day will have scanned only twelve hours of the lunar day. In cases where the rhythm is obviously a lunar-tidal one, with two peaks symmetrically placed in a lunar day, one can synchronize lunar zenith with lunar nadir and thus obtain complete scanning of the now approximately 12-hour day. Alternatively, one can calculate a "residual" diurnal curve and use it to make appropriate corrections on the observed 15-day lunar curve. A residual diurnal curve for each 15-day period was obtained by using the mean diurnal values which include the 15-day period for which the correction was to be

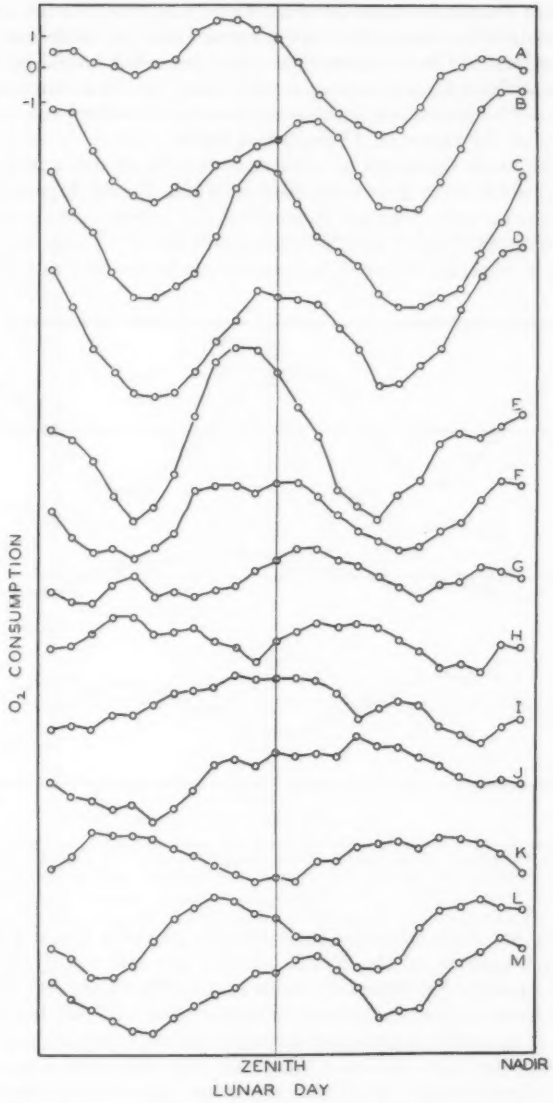

FIGURE 3. Mean lunar-day variation in O_2-consumption of *Uca pugnax* for 13 15-day periods. The letters identify the dates of the periods as found in Table I.

used. The mean diurnal values are repeated as if they represented successive days but are synchronized for lunar time and a mean value for each hour of the lunar day is thus obtained. These values then represent what would be expected if a diurnal rhythm without lunar component were analyzed for a lunar-day rhythm on a 15-day basis. The hourly values thus obtained were subtracted from the corresponding values of the observed 15-day lunar curve.

In Figure 3 is seen the series of corrected mean lunar-day curves for the same 15-day periods which have been described in Table I and Figure 1. It is seen that the first four periods (through September 22) exhibit quite symmetrically bimodal curves with maxima at or near zenith and nadir. There is a tendency for the zenith peak to alternate between an hour or two before and an hour or two after

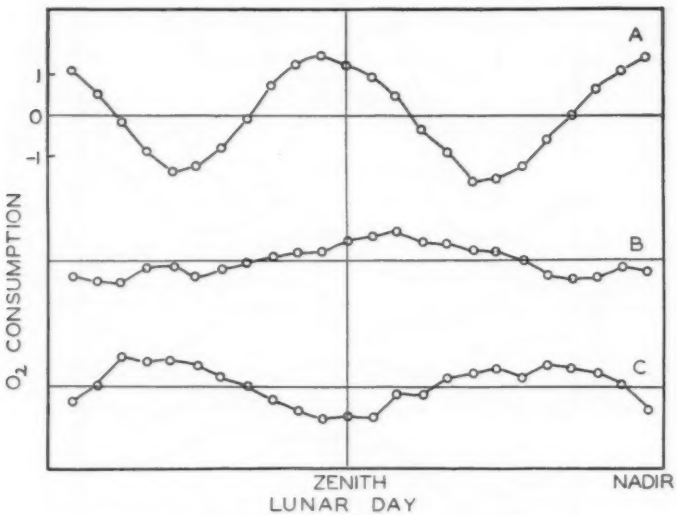

FIGURE 4. Mean lunar-day variation in O_2-consumption of *Uca pugnax* for three periods throughout the year (see text for further explanation).

zenith. During late September and early October (Curves E and F) there is some decrease in the regularity of the curves but they are still clearly bimodal and the peaks are still close to the times of zenith and nadir. Curve G (October 22 to November 6) shows a clear maximum at between one and two hours after zenith; the maximum is smoothly approached during a six-hour period on either side of it. The other twelve hours of the lunar "day" are irregular with apparent secondary maxima about three hours before lunar nadir and about five hours after it. The curves for the periods from November 19 through January 23 (Curves H, I, J) are irregular and of low absolute amplitude although, like the diurnal rhythms, the relative amplitudes show no consistent trend. These three curves on the whole seem to be unimodal in character with high values predominating near the time of lunar zenith. Curve K, Figure 3, representing the period February 2

to 18, is bimodal and in this respect resembles the "summer" curves. However, the phase relations in February are different from those observed in the summertime so that now the minima coincide with lunar zenith and nadir. The final two curves in Figure 3 are those obtained during June and July, 1960; they are bimodal with maxima near the times of zenith and nadir. Here again the tendency toward alternation of peaks around zenith is evident.

In Figure 4 are seen curves summarizing the data of Figure 3. Curve A, Figure 4 represents July 25 to October 25; Curve B is for October 25 to January

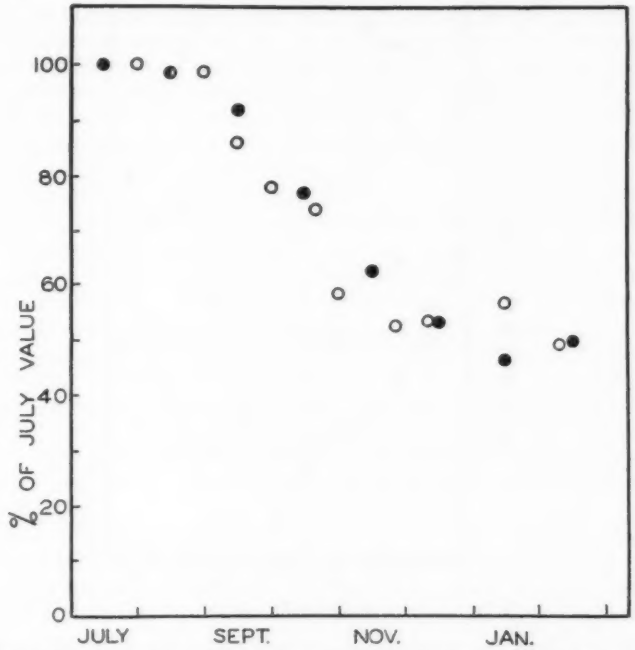

FIGURE 5. Mean monthly outdoor air temperature (solid circles) and mean rates of O_2-consumption of *Uca pugnax* (open circles). (The data on temperature were provided by Dr. Charles Packard of the Marine Biological Laboratory.)

23 and clearly reveals the unimodal nature of the curve for this period; Curve C is for February 2 to 18. From this figure it is clear that three distinct types of lunar day curves are obtained at three different times of the year.

Figure 5 contains data for the mean monthly outdoor air temperature at Woods Hole, Mass., for the months July, 1959, through February, 1960, and the mean rates of O_2-consumption calculated for each 15-day period throughout the same months. In both cases the July value is taken as 100% and the subsequent values are plotted as the appropriate per cent of that value. It is obvious that any two curves drawn through the two sets of points would be practically indistinguishable.

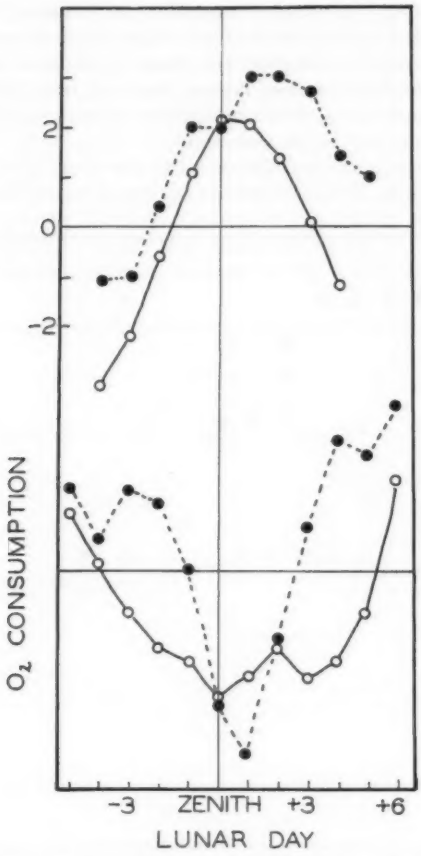

FIGURE 6. Variations in O_2-consumption of *Uca pugnax* for the hours around lunar zenith on four different days.

Figure 6 represents the values obtained, on four different days, during November and December, for the nine-hour period over lunar zenith.

DISCUSSION

The question of the extent to which maintenance of animals in the laboratory contributes to the difference in rates observed in these studies deserves consideration. It will be recalled that collections were made on the following dates: September 30, 1959, January 7, 1960, and January 29, 1960. Therefore, at the end of the test periods October 11 to 25, January 9 to 23, and February 2 to 18, animals had been in the laboratory for 25, 16 and 20 days, respectively. Yet the rates obtained in the

last two periods are considerably lower than those found for the period October 11 to 25. On the other hand, the rates observed in January and February are not significantly different from those observed in the December period. The animals used in December had been in the laboratory for 79 days by the end of the test period. Clearly it is not the length of time in the laboratory that is the major factor in the control of respiratory rates.

An interesting correlation is evident when the mean rates of O_2-consumption for successive periods are compared with mean monthly outdoor air temperature for the corresponding months, as is done in Figure 5. Since the respiratory rates were determined under constant temperature conditions, the remarkable parallel between the two series of points cannot indicate a direct cause and effect relationship. However, the obvious correlation with one factor in the physical environment (which may serve as a representative of a whole complex of annually fluctuating factors), together with the equally obvious lack of correlation with the immediate history of the animals, strongly suggests a contribution of some uncontrolled (and unrecorded) environmental factor to the apparent annual rhythm in respiratory rate.

It is realized that the results reported here are in apparent disagreement with relationships between summer and winter rates of respiration for *Uca pugnax* previously reported by Vernberg (1959) and Teal (1959). In both cases when *Uca* were tested at the same temperatures during summer and winter the rates were higher in the winter time. The animals used by both of these investigators came from different geographical areas than did those used in our experiments. Different methods of measuring O_2-consumption were used, and their methods involved much shorter test times. The maximum time for which their animals were exposed to the measurement situation would be about 8 hours and this only for the lower temperatures. In our case the first 8 to 16 hours of recording were not included in the calculations. Until some of these more obvious differences in conditions have been examined experimentally it seems unprofitable to do more than acknowledge the difference in results.

In addition to the apparent annual cycle in mean rates of O_2-consumption, the results reported here reveal variations in the form of the lunar rhythm. As was seen in Figures 3 and 4, three types of curves were encountered in going from July to February. The curve obtained in the summertime and that for February are both bimodal, one being the inverse of the other; in the intervening period a unimodal curve was obtained.

The apparently unimodal rhythm observed during the transitional period is very probably the result of averaging together for any one period curves, from single days, which show varying degrees of inversion. Evidence bearing on this question was presented in Figure 6 which shows data for the period centering on lunar zenith for four different days. It is quite clear that on two of these days distinct maxima were recorded at lunar zenith while on the other two distinct minima occurred. The relatively few days during the transitional period for which clear inversions are evident can easily be explained when it is realized that even a single record represents the sum of four animals' respiration. As an increasing proportion of the population shows an inversion of the summer pattern at lunar zenith, the chances of getting all the animals being recorded at any one time exhibiting the inverted pattern are increased. This interpretation is supported by the observation that in

February the records show inversion on more than 50% of the days while in less than 25% of the records is there a peak at lunar zenith.

Since the animals tested in December and January show similar patterns for the lunar-day rhythm, although one group has been in the laboratory for 79 days and the other for only 16, it is clear that the conditions of maintenance in the laboratory are not sufficient to explain the observed changes in the rhythm. One condition that was common to all the animals, whether in the laboratory or in the field, was the absence of illumination periods limited to the time of low tide. From early October it was not possible to collect crabs except by digging. Since they remained in their burrows they would not be exposed to all of the changes associated with low tide to which they are exposed in the summertime. These animals would presumably be in continuous darkness. The animals in the laboratory, on the other hand, would have experienced light periods longer than those to which they are normally exposed, and lacking in any tidal component. It is possible, therefore, that the absence of illumination periods associated with the tides permitted this inversion of the form of the rhythm. It is to be noted, however, that there is no evidence of a loss of precision of the period; the majority of the animals are in synchrony during February. The simplest explanation seems to be that the animals receive information concerning the time of lunar zenith and nadir. The difference between summer and winter respiratory patterns might reflect a difference in pattern of the "information" or it might represent a different sign of response on the part of the animals.

Regardless of interpretations as to cause, it is clear from the observations reported here that animals, while showing no obvious changes in the diurnal rhythm, have exhibited alterations in the lunar-day rhythm of respiration. There is no evidence of change in the period of the rhythm but there is evidence of "preferred" phase relations to lunar zenith and nadir.

Summary

1. Records of the O_2-consumption of *Uca pugnax* were obtained during the summer, autumn, and winter months and analyzed for diurnal and lunar-day rhythms.

2. An apparent annual rhythm in mean rate of respiration is reported.

3. The respiratory rate during December, January, and February was found to be approximately 50% of that during June, July, and August. During September, October, and November, a gradual decrease in rate was observed.

4. The form of the diurnal rhythm of respiratory rate showed no changes associated with time of year. Similarly, no seasonal changes in phase relations were observed.

5. The range of both diurnal and lunar-day rhythms showed fluctuations but these were not clearly associated with time of year.

6. The lunar-day rhythm was found to show changes as follows: June through September the curve was bimodal with maxima at lunar zenith and nadir; beginning in October and continuing through January the curve was unimodal with a maximum near lunar zenith; in February the curve was bimodal but with minima at lunar zenith and nadir.

7. The unimodal curve of the transitional period is interpreted as being the resultant curve of a mixed population.

8. The possible relationship of exogenous factors to these seasonal variations is discussed.

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THE JUVENILE HORMONE. II. ITS ROLE IN THE ENDOCRINE CONTROL OF MOLTING, PUPATION, AND ADULT DEVELOPMENT IN THE CECROPIA SILKWORM

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In the history of every hormone there is a more or less prolonged period when the factor is recognizable only as a certain "activity" within a living system. Progress at this stage is largely dependent on the development of a method of biological assay which is simple, selective, and quantitative.

In the case of the juvenile hormone of insects, the pioneeering studies that led to the discovery of the hormone simultaneously directed attention to a method for its assay. This method, as developed by Wigglesworth (1936, 1948, 1958), is performed on mature nymphs of *Rhodnius*. In brief, a fifth instar nymph is given a blood meal to provoke the molt to the adult stage. If one now implants active corpora allata (or, by parabiosis, transfuses blood containing juvenile hormone), the nymph transforms into an adult which retains certain nymphal characteristics. By the use of this assay, Wigglesworth (1940) found the corpus allatum of *Rhodnius* to be active in the immature nymph, inactive in the mature nymph, and active again in the adult.

Numerous investigators, following Wigglesworth's lead, have utilized the "larval assay" in testing the endocrine activity of corpora allata. The literature includes studies of the following genera: *Bombyx* (Bounhiol, 1938; Fukuda, 1944; Ichikawa and Kaji, 1950), *Dixippus* (Pflugfelder, 1939, 1958), *Tenebrio* (Radtke, 1942), *Galleria* (Piepho, 1942, 1950b), *Melanoplus* (Pfeiffer, 1945), *Drosophila* (Vogt, 1946), *Gryllus* (Poisson and Sellier, 1947), *Oncopeltus* (Novák, 1951), and *Calliphora* (Possompès, 1953). The conclusions derived in all these studies have confirmed the fact that the corpora allata undergo substantial changes in activity during the course of metamorphosis.

At the Harvard laboratory we also have tried to make use of the larval assay in testing for juvenile hormone. A survey of all of our experiments performed during the past fifteen years fails to reveal a single instance in which the implantation of active corpora allata interfered with the transformation of fifth stage Cecropia larvae into normal pupae. For reasons that are not fully understood, the larval assay does not work in the case of the Cecropia silkworm.

Solution of our problem came from an unexpected direction. As described in the previous paper of this series, the "pupal assay" was accidentally discovered in 1947; unlike the larval assay, it proved to be an extremely sensitive test for juvenile hormone (Williams, 1952a, 1959).

In the present study the pupal assay has been used as a principal tool in a study

[1] This study was supported, in part, by Grant RG-5920 from the National Institutes of Health.

of the role of juvenile hormone in the growth and metamorphosis of the Cecropia silkworm.

Material and Methods

The experiments were performed on larvae, pupae, and adults of the Cecropia silkworm, and on pupae and adults of the Cynthia silkworm. In addition to methods described previously, the following procedures were utilized.

1. *The pupal assay for juvenile hormone*

Pairs of corpora allata-corpora cardiaca complexes were excised from larval, pupal, or adult donors, and placed in Ringer's solution. In the early experiments the corpora allata were dissociated from the closely attached corpora cardiaca. This tedious maneuver proved inconsequential (Williams, 1959) and was generally omitted in later experiments.

The glands were tested for endocrine activity by implanting them into the abdomens of female Cecropia pupae that had been stored at 6° C. for at least four months. For reasons considered below, pupae that had already initiated adult development were not suitable as test animals. The implantation was accomplished as follows:

Each test pupa was anesthetized with carbon dioxide, and a disc of integument excised from the tip of its abdomen. By means of forceps, one to eight corpora allata were placed among the strands of fat-body deep inside the abdomen. Crystals of an equal part mixture of phenylthiourea and streptomycin sulfate were spread in the wound, along with sufficient Ringer's solution to fill the body cavity. The wound was capped with a plastic window which was sealed in place with melted wax. Finally, the pupa was returned to air and stored at 25° C.

Within a few days the wound was healed by a deposit of blood cells on the plastic window and the centripetal spread of the simple epidermis from around the margins of the wound. Adult development was ordinarily initiated after about ten days; the zero day of development was recognized by the beginning of the retraction of the regenerate epidermis underlying the plastic window (Schneiderman and Williams, 1954).

On about the fifth day of development the first indication of a positive test for juvenile hormone was evident under the window. Here one could witness the formation of a new pupal cuticle which soon became tan and sclerotized. A few days later, the old pupal cuticle became thin and crisp, due to a precocious activation of the molting-fluid and the localized resorption of endocuticle (Passonneau and Williams, 1953). Commonly, one could detect the discharge of a white sludge of meconium into the molting-fluid—an event that has rarely been observed in the course of normal metamorphosis.

Development was allowed to proceed for a total of twenty-one days or until the molting-fluid was partially resorbed. The pupal exuvia was then peeled away with forceps. The insect was immersed in Ringer's solution and subjected to detailed external and, in many cases, internal examination.

A positive test for juvenile hormone is signaled by the preservation of pupal characters (Williams, 1952a, 1956, 1959)—a finding which will be considered in detail in the section on Results.

2. Excision of larval corpora allata

Matched pairs of larvae were sacrificed and the corpora allata dissected from their heads, as previously described in the case of adult corpora allata (Williams, 1959, page 327). In certain experiments it was necessary to excise the corpora allata without killing the larval donors. By adaptations of a method suggested by Dr. William Van der Kloot (unpublished observations), a surgical approach through the underside of the neck was utilized, as follows:

A 3-cm. length of dowel was attached to a small base-board so that the dowel stood vertically above the perforated plate of the anesthesia funnel. The top end of the dowel was grooved to fit the dorsum of the larval head capsule. The larva was deeply anesthetized and placed in the anesthesia funnel so that the underside of the neck was stretched and flexed over the top end of the dowel. The head capsule was held in the groove by small clips so that the thorax and anterior abdominal segments hung vertically. In this way the blood was pressed from the neck region and the latter was flattened and essentially bloodless.

The ventral midline of the neck was lifted with forceps and a single V-shaped incision was made through the integument with microscissors. Under a dissecting microscope the operation was carried out through the incision, first on one side of the neck and then on the other. With blunt probes the muscles of the neck were pressed apart and the corpora allata located and excised.

At the conclusion of the operation the flap of skin was spread in place. The animal was stored in a capped cardboard container at 5° C. until the next day. It was then returned to room temperature and placed on a netted branch of wild-cherry leaves.

3. Excision and transplantation of wing-discs

The thorax plus first abdominal segment was isolated, opened along the mid-dorsal line, and spread and pinned under Ringer's solution. After the removal of the viscera, the two pairs of wing-discs were located between the body wall and the complex musculature of the meso- and metathorax.

Each disc, together with its peripodal sac, was trimmed away from surrounding structures and placed in a dish of insect Ringer. In some cases the dissection was continued by cutting away the peripodal sac. The discs were implanted under abdominal windows of previously chilled pupae, as described above.

Experimental Results

1. Quantitative aspects of the pupal assay for juvenile hormone

When a living, active corpus allatum is implanted into a test pupa, the gland survives and becomes the site of synthesis and secretion of juvenile hormone. If several active glands are implanted, a corresponding number of synthetic centers are established. So, in theory, one should be able to vary the rate of secretion and accumulation of juvenile hormone by varying the number of implanted glands. The developmental reactions of the test animal should then reflect the quantitative aspects of the pupal assay.

In experiments of this type, advantage was taken of the high activity that one routinely observes in tests of the corpora allata of freshly emerged Cecropia moths

(Williams, 1959). Four (two pairs) adult corpora allata were implanted into each of 24 test pupae; a second group of 24 pupae each received only a single implant (one-half pair). The hosts were then placed at 25° C. to await the onset of development.

Three to four weeks later, a spectacular difference was evident between the two groups of animals. The individuals that received the four implants showed a generalized inhibition of adult differentiation, as signaled by the formation of a new pupal cuticle throughout broad areas of head, thorax, and abdomen. Indeed, some of these animals could properly be described as second pupal stages in which only

TABLE I

Developmental status of previously chilled Cecropia pupae after receiving implants of corpora allata. The endocrine activity of the implants is scored in intensities ranging from zero to five

Intensity of reaction	Anatomical characters			
	Head	Thorax	Abdomen	Internal anatomy
0	Adult	Adult	Adult	Adult
1	Normal adult, except for small facet-free crescents in eyes*	Adult with pupal cuticle occasionally in midline of prothoracic tergum*	Adult except for small zone of pupal cuticle at base of genitalia*	Adult
2	Normal adult, except for facet-free crescents in eyes	Pupal cuticle in midline of tergum	Zones of pupal cuticle anterior to intersegmental membranes; genitalia slightly arrested with localized pupal cuticle	Adult with slight suppression of gonad development
3	Covered with pupal cuticle with pubescence only on frons; antennae show incomplete barbs and pupal cuticle locally on shaft	Large zones of pupal cuticle on tergum and legs; wings show scales, but incomplete pigmentation	Generally covered with pupal cuticle showing sparse pubescence; genitalia very inhibited and covered with pupal cuticle	Gonads show considerable arrest; flight muscles show only early development; gut retains pupal configuration; prothoracic glands persistent
4	Pupal cuticle on head, antennae, and palps; antennae show only traces of segmentation or of subdivision into barbs	Pupal cuticle throughout with only localized islands of pubescence; wings generally white, fleshy and friable	Pupal cuticle throughout; genitalia show no development	Only traces of development in gonads, gut, and muscles; fat-body friable and in chunks; prothoracic glands persistent
5	Pupal throughout except for small pigmented eyes	Pupal cuticle throughout; sometimes also at base of wings and around margins of forewings	Wholly pupal	Pupal; prothoracic glands persistent

* Pupal cuticle forms in all zones where the integument has been injured.

traces of adult characteristics had been differentiated. By contrast, the pupae that had received only a single corpus allatum ordinarily developed into adult moths which showed few abnormalities except for the formation of a new pupal cuticle under the plastic window where the pupal integument had been excised in the implantation procedure.

These results have been duplicated and extended on a large scale during the past fourteen years. Under most conditions and circumstances, the standardized pupal assay has proved to be a sensitive, selective, and semi-quantitative test for the concentration of juvenile hormone.

Under the standardized conditions of the assay, each concentration of juvenile hormone ordinarily produces a certain pattern of inhibition. In practice, it was possible to subdivide the intensities of the reactions into six categories. This scoring system, summarized in Table I, permitted the pupal assay to be used in a study of corpus allatum activity at successive stages in the life history of the Cecropia silkworm.

2. *Endocrine activity of corpora allata*

A. Larval corpora allata

Corpora allata were removed from matched pairs of Cecropia larvae at stages ranging from the middle of the third instar to the end of the fifth instar. The two pairs of glands were, in each case, implanted into a single female test pupa, as described under Methods.

Before considering the results of the assays, it is worth recalling that the growth and metamorphosis of the corpora allata are synchronized with the growth and

TABLE II

Activity of Cecropia corpora allata implanted into chilled Cecropia pupae (2 pairs of glands into each test pupa)

Stage of donors	No. of tests	Intensity of hormone reaction						Average Index*
		0	1	2	3	4	5	
3d instar	5	1	2	0	2	0	0	1.6
Late 3d	2	0	1	1	0	0	0	1.5
3d molting	9	0	3	5	1	0	0	1.8
Early 4th instar	11	0	2	2	6	1	0	2.5
4th	6	1	1	2	2	0	0	1.8
Late 4th	6	1	2	2	1	0	0	1.5
4th molting	10	1	5	2	2	0	0	1.5
Early 5th instar	12	0	4	0	7	1	0	2.4
5th	19	4	9	3	3	0	0	1.3
Late 5th	13	2	2	6	3	0	0	1.8
1st day spinning	19	2	15	2	0	0	0	1.0
2nd day spinning	12	2	10	0	0	0	0	0.8
Finished spinning	7	0	6	2	0	0	0	1.1
Early prepupa	10	4	6	0	0	0	0	0.6
Fresh pupa	10	10	0	0	0	0	0	0
Unchilled pupa	7	7	0	0	0	0	0	0
Chilled pupa	29	29	0	0	0	0	0	0
1–2 day adult development	5	5	0	0	0	0	0	0
9–11 day	6	6	0	0	0	0	0	0
14th day	18	13	1	2	2	0	0	0.6
17th day	4	3	1	0	0	0	0	0.25
Adult (freshly emerged)	24	0	0	2	5	12	5	3.8

* For definition, see text.

metamorphosis of the animal as a whole (for review see Pflugfelder, 1958). And since four glands were routinely implanted into each test pupa, the assay did not compensate for the changing mass of endocrine tissue. What the pupal assay recorded was the endocrine activity of the four implants, irrespective of size, rather than the activity per unit mass of endocrine organ.

Over a period of several years a total of 131 assays were performed on larval corpora allata. In the results tabulated in Table II, a certain scatter is observed in the activity recorded at each stage. In order to derive an overall index of activity

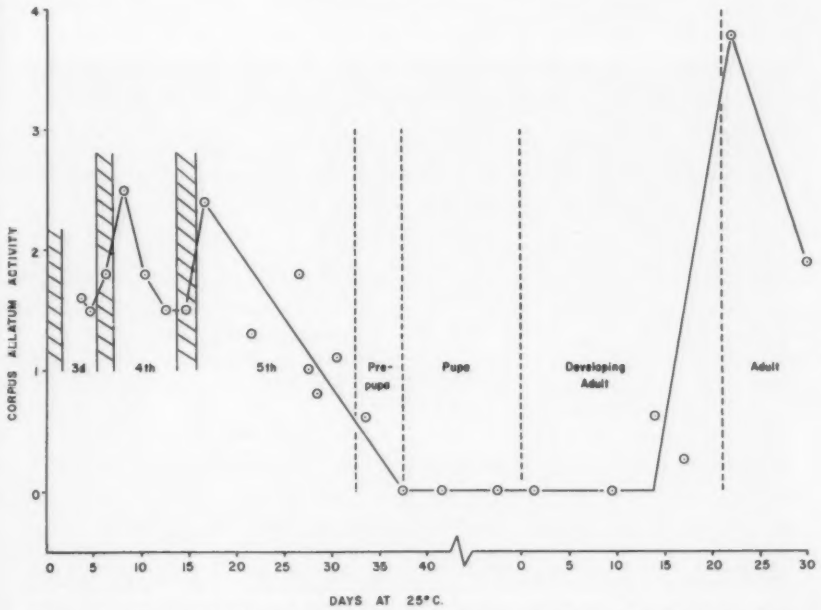

FIGURE 1. Changes in the endocrine activity of the corpora allata of the Cecropia silkworm during the third, fourth, and fifth instars of larval life and during metamorphosis. The average indices of corpus allatum activity (derived from "pupal assays") are plotted as a function of the stage of development of the donor animals. The discontinuity in the x-axis signals the storage of donor pupae at 6° C. for 10 to 20 weeks; the cross-hatched zones correspond to the periods of larval molting.

at each stage, a zero response was counted as zero, a one response as one, a two as two, and so on. The total was then divided by the number of experimental animals to yield an "average index" of corpus allatum activity at each stage.

In the plot of these average indices in Figure 1, attention is first directed to what appears to be a cycle of corpus allatum activity during the third and fourth larval instars. Their endocrine activity is apparently minimal just prior to the molt, maximal shortly after the molt, and subject to a steady decline during the instar itself. As illustrated in Figure 1, this decline is particularly striking during the final (fifth) larval instar.

Late in the fifth instar, when the activity of the corpora allata has declined to a critically low level, a dramatic change occurs in the silkworm's behavior. It ceases to feed, empties its gut, and then begins a two-day period of spinning. This impressive change in behavior is the earliest sign of approaching pupation.

B. Prepupal corpora allata

The onset of the prepupal stage is signaled by a detachment and retraction of the epidermis from the larval cuticle and by the initiation of the cytological events which, during a period of five days, transform the larva into a pupa. In Figure 1 we note the surprising finding that the corpora allata show a low but definite activity during the prepupal period—a result which will be considered below in further detail.

C. Pupal corpora allata

The decline in corpus allatum activity continues during the prepupal period. Then, immediately after the pupal ecdysis, the glands for the first time are found to be completely inactive.

As illustrated in Figure 1, the inactivity of pupal corpora allata persists during the entire pupal period, irrespective of whether the pupae are stored at high or low temperatures. In my entire series of assays I have never obtained a positive test for the pupal corpora allata of Cecropia, Polyphemus, Cynthia, or any other lepidopteran. In a univoltine species such as Cecropia, the pupal stage extends from midsummer until the following spring. So, during a period of about eight months, the corpora allata are apparently inactive.

D. Corpora allata during adult development.

Adult development, once initiated, requires three weeks at 25° C. The corpora allata continue to be inactive during the first two weeks of this period. On the fourteenth day of development one begins to record renewed activity in the glands of a certain proportion of individuals (Table II and Fig. 1). Within the final week of adult development the corpora allata recover their endocrine activity. Indeed, in the pupal assay the glands of freshly emerged male or female moths show higher activity than at any other stage in the life history.

In the absence of functional mouth-parts, the Cecropia moth lives for only 7 to 10 days at 25° C. During this brief period the glands of most individuals undergo a marked decline in activity to a level approximately one-half of that a week earlier.

3. *The role of juvenile hormone in pupation*

As noted in Section 2B, the corpora allata show a low but definite activity during the prepupal period. This is an extremely surprising result since the prepupal stage is distinguished by metamorphic changes on an unprecedented scale. The experiments, about to be described, were designed to test the physiological significance of the low concentration of juvenile hormone that is apparently present during the prepupal period.

In an initial series of experiments performed in collaboration with Dr. William H. Telfer, the corpora allata were excised from a group of eight Cecropia larvae early in the fifth instar. The loss of the glands seemed inconsequential. Feeding and growth continued and two weeks later the animals spun normal cocoons. But, within the following ten days, only two of the eight animals transformed into normal pupae. The other six formed strange creatures in which a considerable number of tissues and organs had overleaped the pupal stage by undergoing precocious adult differentiation.

One of these animals is illustrated in Figure 2. The head shows the pigmented, faceted, compound eyes of the adult. The antennae exhibit the segmentation and

FIGURE 2. By excision of the corpora allata during the final larval instar, this animal was caused to undergo pupation in the absence of juvenile hormone. A large number of larval tissues and organs have undergone precocious adult differentiation without traversing the pupal stage.

subdivisions characteristic of early adult development. The thorax is covered with a mixture of rugose pupal cuticle and smooth cuticle of the adult type. The adult patagia have developed at the base of the fore-wings. The sclerotization of the thoracic tergum is adultoid. The thoracic pleura and sternum are covered for the most part by a smooth, adult-type cuticle. The legs show segmentation and the differentiation of tarsal claws and pulvilli. The proximal ends of the wings are covered by adult cuticle. The cuticle of the abdomen is wholly pupal, except in the immediate region of the genitalia; the latter are represented, not by imaginal discs, but by miniature adult genitalia which show an early elaboration of the various valves and adult structures. Dissection revealed that the fat-body was similar to that of a pupa after the initiation of adult development. Moreover, the ovaries

TABLE III

Effects of removal of corpora allata from fifth instar Cecropia larvae

State at time of operation	No. of animals	Results	
		Normal pupa	Pupal-adult
Young 5th instar	3	0	3
Mid 5th instar	9	5	4
Late 5th instar	5	2	3
Spinning	13	12	1

showed the differentiation of ovarioles to a stage corresponding to that encountered in early adult development.

The experiment was repeated on a larger scale utilizing animals at successive stages in the fifth instar. The results in Table III record the production of pupal-adult mixtures in all three animals from which the corpora allata were extirpated early in the fifth instar. Excision in the mid or late fifth instar gave rise to mixed forms in about half the animals. And when the operation was further postponed to the period of spinning, only one of thirteen animals developed into a mixed form.

These findings suggest that the juvenile hormone plays a definite role in the endocrine control of pupation. Evidently, the low concentration of juvenile hormone in the mature larva and prepupa is necessary to prevent the precocious adult differentiation of larval tissues and organs.

4. *Precocious adult differentiation of larval wing-discs*

Wing-discs were excised from fourth and fifth instar Cecropia larvae and implanted into previously chilled Cynthia pupae just before the latter initiated adult development. In this manner the discs were exposed to the ecdyson of the developing host under conditions where juvenile hormone was absent. After the hosts had completed adult development, the implants were recovered and subjected to detailed examination.

As is true of implants of all epidermal tissues, the implants took the form of cyst-like structures with the integumentary surface facing inward. Each cyst was cut open and a record made as to whether it had formed a rugose pupal cuticle or a smooth, scale-covered cuticle of the adult type.

TABLE IV

Metamorphosis of larval wing-discs implanted into previously chilled pupae

Initial status of discs	No. of discs	No. of hosts	Differentiation of implants		
			Pupal	Pupal-adult	Adult
4th instar	20	10	5	8	7
Early 5th	2	2	0	2	0
Late 5th	9	4	4	1	5
Spinning 5th	4	2	0	4	0

The results are summarized in Table IV. Of the 35 wing-discs that were studied, 12 showed adult cuticle, 9 showed pupal cuticle, and 15 showed a mixture of pupal and adult cuticle. Certain cysts showed the differentiation of the articular sclerites distinctive of the adult wings.

It is noteworthy that precocious adult differentiation was readily obtained in wing-discs as early as the fourth instar. Consequently, when exposed to ecdyson in the absence of juvenile hormone, these implants omitted the fifth larval instar as well as the entire pupal stage. Here again we see that a certain low concentration of juvenile hormone is prerequisite for the transformation of a Cecropia larva into a normal pupa.

These results are reminiscent of Schaller's (1952) finding that larval honeybees undergo precocious adult development when decapitated prior to pupation. Moreover, Nayar (1954) has reported that pieces of larval integument undergo precocious adult differentiation when transplanted to pupae of *Ephestia, Galleria,* or *Pieris.* Here again we see that in many species a certain low concentration of juvenile hormone is necessary for the normal transformation of a larva into a pupa. This fact has long been suspected (Piepho, 1951; Wigglesworth, 1954, 1959; Schneiderman and Gilbert, 1959; Novák and Červenková, 1960), but is documented for the first time in the present investigation.

Discussion

1. *Juvenile hormone and adult development*

The experimental results direct attention to a prolonged period during which the corpora allata appear to be totally inactive in the secretion of juvenile hormone. This period begins immediately after pupation and continues throughout the entire pupal stage. Since the prothoracic glands are also inactive at this same time (Williams, 1952b) the pupal stage is characterized by subthreshold titers of both ecdyson and juvenile hormone.

After eight months of pupal diapause, the prothoracic glands recover their endocrine activity. Ecdyson is secreted and adult development begins. Meanwhile, the corpora allata continue to be inactive—a condition which persists throughout the first two-thirds of adult development.

The pupal assay derives its sensitivity from the fact that the early phase of adult development takes place only if juvenile hormone is absent. If the hormone is supplied by the implantation of active corpora allata, the net result is to block adult differentiation and to promote the formation of a second pupal instar. In effect, we duplicate the endocrine conditions peculiar to larval life in that ecdyson and juvenile hormone are caused to act side-by-side. The pupa molts into a further pupal instar just as, under the same circumstances, the larva molts to a further larval instar.

2. *Juvenile hormone and pupation*

The endocrine stimulus for pupation is the action of ecdyson in the presence of a low but finite titer of juvenile hormone. If the corpora allata are excised so that the larva approaches pupation in the absence of juvenile hormone, then a large number of larval tissues overleap the pupal stage and undergo precocious adult develop-

ment. The same result was observed when larval wing-discs were subjected to *in vivo* culture in the presence of ecdyson and the absence of juvenile hormone. Here again we see that the presence of a low concentration of juvenile hormone at the time of pupation serves as a brake on the precocious acting-out of the life-plan.

3. *Juvenile hormone and larval development*

According to the pupal assays, the corpora allata undergo a cycle of endocrine activity during successive larval instars. If the glands are extirpated so that an immature larva approaches a larval molt in the absence of juvenile hormone, the result, once again, is precocious metamorphosis in response to ecdyson (Bounhiol, 1938; Piepho, 1943; Fukuda, 1944; Williams, 1946).

In Figure 1 it is of particular interest to note that the corpora allata show a marked decline in activity during the intermolt period and are minimally active just prior to the molt itself. By virtue of the declining titer of juvenile hormone, one would anticipate some measure of progressive differentiation between successive larval instars.

This inference is confirmed by the striking heteromorphic changes (Snodgrass, 1954) which the Cecropia silkworm undergoes during the larval period. Thus, the first instar is jet black and covered with spinose tubercles; the second instar is yellow with black tubercles and spots; the third instar is greenish yellow with black spots and red, blue, and yellow spinose tubercles; the fourth instar is bluish green with pairs of red, yellow, and blue spinose tubercles; the fifth instar is bright green, shows distinctive red and yellow tubercles on the thoracic segments and a considerable reduction in the spines on all tubercles. In summary, each larval instar shows distinctive characteristics which, irrespective of the insect's overall size, permit one to recognize the individual instars with ease. It is no exaggeration to say that the magnitude of these changes between successive larval instars equals or exceeds those commonly encountered in hemimetabolous insects at the time of metamorphosis.

If larval heteromorphosis is attributable to the declining titer of juvenile hormone prior to the larval molts, then, by augmenting the concentration of juvenile hormone, one at least in theory should be able to obtain fifth stage larvae which retain the form of the first stage. Such experiments have not been performed. However, according to the unpublished observations of Dr. Judith Willis, the Cecropia silkworm, under certain environmental conditions, repeats the second, third, or fourth larval instar without change in form. Presumably, in all these cases, the corpora allata fail to undergo the normal decline in activity prior to the extra molt.

As pointed out by Snodgrass (1954), spectacular degrees of larval heteromorphosis are frequently encountered among predatory or parasitic species of insects, examples being known among the Neuroptera, Coleoptera, Strepsiptera, Lepidoptera, Hymenoptera, and Diptera. Evidently, the heteromorphic larval molts are here preceded by an even greater decline in corpus allatum activity than that encountered in the Cecropia silkworm.

4. *Theory of juvenile hormone action*

A comprehensive theory of the action of juvenile hormone must account for the following findings: (1) Ecdyson is a potent growth hormone either in the presence

or absence of juvenile hormone. (2) In all of the phenomena here under consideration, juvenile hormone is active only in the presence of ecdyson. (3) When juvenile hormone is absent, ecdyson promotes, not only the synthetic acts prerequisite for growth, but also the new synthetic acts that are necessary for metamorphosis. (4) When ecdyson acts in the presence of juvenile hormone, growth continues, but new synthetic acts are blocked to a degree proportional to the concentration of juvenile hormone.

From this summary we learn that the role of juvenile hormone is to modify the cellular reactions to ecdyson. It appears to do so by opposing progressive differentiation without interfering with growth and molting in an unchanging state. In some unknown manner, it blocks the de-repression and de-coding of fresh genetic "information" without interfering with the acting-out of information already at the disposal of the cells.

5. *The cytological actions of juvenile hormone*

Though we are unable to state how juvenile hormone controls the flow of fresh genetic information, certain clues may be derived from the data already at hand. Thus, in the case of the pupal-adult transformation, it is clear that the events which are sensitive to juvenile hormone take place at the very outset of adult development. So, if the implantation of corpora allata is delayed until the fifth day of the twenty-one days of adult development, it is already too late for juvenile hormone to have any effect. To be maximally effective, juvenile hormone must be present during the initiation of adult development; *i.e.*, during precisely the period when ecdyson is secreted by the prothoracic glands and acts throughout the pupa.

Evidently, the targets of juvenile hormone are certain very early events, including mitotic divisions, which are the normal cellular reactions to ecdyson at the outset of adult development. Whatever these cellular or subcellular events may be, we can state that they occur early, that they show a rapid loss of sensitivity to juvenile hormone, and that, if unopposed by juvenile hormone, they commit the cells to developmental reactions accompanied by metamorphosis.

It is worth recalling that, even at the outset of adult development, the various pupal tissues show a great range of sensitivities to juvenile hormone (Table I). Consequently, a cytological and cytochemical comparison between tissues of high and low sensitivity may yield additional information as to the mode of action of juvenile hormone. This approach seems particularly cogent in the case of the pupal epidermis whose sensitivity to juvenile hormone is vastly amplified at the site of an integumentary injury.

Summary

1. By means of a standardized "pupal assay" for juvenile hormone, the endocrine activity of the corpora allata was found to undergo large and systematic changes during the postembryonic development of the Cecropia silkworm.

2. In each of the larval instars that were studied, the glands are least active just prior to the larval molt and most active shortly after the molt. Larval molting therefore appears to take place in the presence of a declining titer of juvenile hormone—an endocrine situation which apparently permits the striking changes in

morphology and pigmentation which occur in successive larval stages of the Cecropia silkworm.

3. The corpora allata show low but definite activity at the time of pupation. If the glands are extirpated so that pupation occurs in the absence of juvenile hormone, many larval tissues overleap the pupal stage and undergo precocious adult differentiation. Therefore, the low concentration of juvenile hormone in the mature larva plays a definite role in the endocrine control of pupation.

4. The corpora allata are inactive throughout the entire pupal stage. This inactivity persists during the first two-thirds of adult development. During the final week of adult development, the glands recover their endocrine function and are maximally active by the time of emergence of the adult moth.

5. The absence of juvenile hormone proves to be an obligatory feature of the initial phase of adult differentiation. If active corpora allata are implanted into a pupa so that adult development is initiated in the presence of juvenile hormone, the pupa develops and molts into a creature which is a mixture of pupa and adult. The higher the titer of juvenile hormone, the more extensive is the preservation of pupal characters.

6. Juvenile hormone is effective in blocking adult differentiation only when it is present during the first five days of adult development. Consequently, the target of juvenile hormone appears to be certain early events which are the normal reactions to ecdyson at the outset of adult development. If unopposed by juvenile hormone, these events commit the cells to developmental reactions accompanied by metamorphosis.

7. A comprehensive theory is presented for the action of juvenile hormone in the Cecropia silkworm. According to this theory, juvenile hormone modifies the cellular reactions to ecdyson by opposing the de-repression, de-coding or acting-out of fresh genetic information prerequisite for progressive differentiation but not prerequisite for growth in an unchanging state.

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